Anticipated Classification of this Application:
Class 424 Subclass 093.210

Prior Application:
Examiner A. M. Baker
Group Art Unit 1632

Attorney's Docket No. 60435-A/JPW/MMM

HONORABLE ASSISTANT COMMISSIONER FOR PATENTS
Washington, D.C. 20231

August 22, 2000

S I R:

This is a request for filing a X CONTINUATION ____ DIVISIONAL ____ CONTINUATION-IN-PART application under X 37 C.F.R. §1.53(b) ____ 37 C.F.R. §1.53(d),[1] of pending prior application Serial No. 08/862,438 filed on May 24, 1997 of

Edmund La Gamma et al. for
Inventor(s)

METHOD OF PRODUCING GENETICALLY MODIFIED ASTROCYTES AND USES THEREOF
Title of Invention

1. ____ Enclosed is a copy of the prior application, as originally filed and an affidavit or declaration verifying it as a true copy.

2. X A verified statement to establish small entity status under 37 C.F.R. §1.9 and 1.27

   ____ is enclosed.

   X was filed in the prior application and such status is still proper and desired (37 C.F.R. §1.28(a)).

3. X The filing fee is calculated as follows:

CLAIMS AS FILED, LESS ANY CLAIMS CANCELLED BY AMENDMENT

	NUMBER FILED		NUMBER EXTRA*		RATE SMALL ENTITY	RATE OTHER ENTITY		FEE SMALL ENTITY	FEE OTHER ENTITY
Total Claims	15-20	=	0	X	9	18	=	$ 0	$
Independent Claims	1-3	=	0	X	39	78	=	$ 0	$
Multiple Dependent Claims Presented: ____ Yes X No					130	260	=	$ 0	$
					BASIC FEE			$ 345	$
					TOTAL FEE			$ 345	$

*If the difference in Col. 1 is less than zero, enter "0" in Col. 2.

[1] Filing an application pursuant to this section expressly abandons the parent application.

Applicants: Edmund La Gamma et al.
Serial No.: Not Yet Known
Filed: Herewith

August 22, 2000

4. __X__ The Commissioner is hereby authorized to charge payment of the following fees associated with this application or credit any overpayment to Deposit Account No. 03-3125.

__X__ Any additional filing fees required under 37 C.F.R. §1.16.

__X__ Any patent application processing fees under 37 C.F.R. §1.17.

_____ The issue fees set forth in 37 C.F.R. §1.18 at or before mailing of the Notice of Allowance, pursuant to 37 C.F.R. §1.311(b).

5. __X__ Three copies of this sheet are enclosed.

6. __X__ A check in the amount of $ 345.00 is enclosed.

7. _____ Cancel claims ____________________.

8. _____ Amend the specification by inserting before the first line the sentence: --This is a ___continuation ___division of application Serial No.__________, filed __________.--

9. _____ ___ Sheet(s) of ___ informal ___ formal drawing(s) is/ are enclosed.

10. _____ Transfer the drawings from the prior application to this application and abandon said prior application as of the filing date accorded this application. A duplicate copy of this sheet is enclosed for filing in the prior application file.

11. _____ Priority of application No. __________ filed on ____________________ in _______________ (country) is claimed under 37 U.S.C. §119.

_____ The certified copy of the priority application has been filed in prior application Serial No. _______________, filed _______________.

12. __X__ The prior application is assigned of record to The Research Foundation of State University of New York (copy attached)

13. __X__ A preliminary amendment is enclosed.

14. __X__ The power of attorney in the prior application is to:

John P. White (Reg. No. 28,678); Christopher C. Dunham (Reg. No. 22,031); Norman H. Zivin (Reg. No. 25,385); Jay H. Maioli (Reg. No. 27,213); William E. Pelton (Reg. No. 25,702); Robert D. Katz (Reg. No. 30,141); Peter J. Phillips (Reg. No. 29,691); Wendy E. Miller (Reg. No. 35,615); Richard S. Milner (Reg. No. 33,970); Robert T. Maldonado (Reg. No. 38,232); Paul Teng (Reg. No. 40,837); Richard F. Jaworski (Reg. No. 33,515); Elizabeth M. Wieckowski (Reg. No. 42,226); Pedro C. Fernandez (Reg. No. 41,741); and Gary J. Gershik (Reg. No. 39,992)

Applicants: Edmund La Gamma et al.
Serial No. Not Yet Known
Filed: Herewith

August 22, 2000

(a) ____ The power appears in the original papers in the prior application.

(b) X Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.

(c) X Address all future communications to: (May only be completed by applicant, or attorney or agent of record.)

John P. White
Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036

15. X Also enclosed Express Mail Certificate of Mailing No. EL066381097US

16. ____ I hereby verify that the attached papers are a true copy of prior application Serial No.__________ as originally filed on ____________________.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statement and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

8/22/00
Date

Signature John P. White, Reg. No. 28,678

____ INVENTOR(S)
____ ASSIGNEE OF COMPLETE INTEREST
X ATTORNEY OR AGENT OF RECORD
____ FILED UNDER 37 C.F.R. §1.34(a)

Address of Signator:

Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036

08-23-00

A

Dkt. 60435-A/JPW/EMW/MMM

JC868 U.S. PTO 09/643334 08/22/00

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Edmund La Gamma et al.

Serial No.: Not Yet Known

Filed : Herewith

For : METHOD OF PRODUCING GENETICALLY MODIFIED ASTROCYTES AND USES THEREOF

1185 Avenue of the Americas
New York, New York 10036
August 22, 2000

Assistant Commissioner for Patents
Box Patent Application
Washington, D.C. 20231

Sir:

EXPRESS MAIL
CERTIFICATE OF MAILING
FOR THE ABOVE-IDENTIFIED APPLICATION

"Express Mail" mailing label number: EL066381097US

Date of Deposit: August 22, 2000

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. §1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.

Printed Name: Jamel Khass

Respectfully submitted,

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Dkt. 60435-A/JPW/MMM

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Edmund La Gamma et al.
Serial No.:	Not Yet Known
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For :	METHOD OF PRODUCING GENETICALLY MODIFIED ASTROCYTES AND USES THEREOF

1185 Avenue of the Americas
New York, New York 10036
August 22, 2000

Assistant Commissioner for Patents
Washington, D.C. 20231

Box Patent Application

Sir:

PRELIMINARY AMENDMENT

Applicants request that the following amendments be made in the above-identified application.

In the Specification:

Page 1, after the title and before the first line of the specification, please add the following new paragraphs:

> --This application is a continuation of U.S. Serial No. 08/862,438, filed May 24, 1997, now U.S. Patent No. 6,106,827 to issue on August 22, 2000, which is a continuation of U.S. Serial No. 07/909,281, filed July 6, 1992, now abandoned, the contents of which are hereby incorporated by reference.--

In the claims:

Please cancel claims 1-64 without disclaimer or prejudice to applicants' right to pursue the subject matter of these claims at a later date in a continuation or divisional application.

Please add new claims 65-79 as follows:

65. (New) A non-virally genetically modified non-tumorous astrocyte comprising: DNA consisting of a first DNA encoding a selectable marker and a second DNA encoding a biologically active molecule; wherein expression of the DNA encoding the selectable marker is regulated by a promoter; and wherein expression of the DNA encoding the biologically active molecule is regulated by a regulatory element for controlling expression of said DNA, said regulatory element including a regulatable promoter which controls expression in said astrocyte, and wherein said first and second DNA, said promoter and said regulatory element are stably incorporated into the genomic DNA of said astrocyte.

66. (New) The genetically modified astrocyte of claim 65 wherein said selectable marker is a protein conferring neomycin resistance.

67. (New) The genetically modified astrocyte of claim 65 wherein said selectable marker is a protein conferring methotrexate resistance.

68. (New) The genetically modified astrocyte of claim 65 wherein expression of said DNA encoding said biologically active molecule results in the production of a protein.

69. (New) The genetically modified astrocyte of claim 65 wherein said biologically active molecule is a growth factor.

70. (New) The genetically modified astrocyte of claim 65 wherein said biologically active molecule is a cytokine.

71. (New) The genetically modified astrocyte of claim 65 wherein said biologically active molecule is tyrosine hydroxylase.

72. (New) The genetically modified astrocyte of claim 65 wherein said regulatable promoter is an inducible promoter.

73. (New) The genetically modified astrocyte of claim 72 wherein said inducible promoter is a human preproenkephalin promoter.

74. (New) An astrocyte cell line resulting from the genetically modified astrocyte of claim 65.

75. (New) The astrocyte of claim 65 wherein the promoter regulating expression of DNA encoding the selectable marker is the thymidine kinase promoter.

76. (New) The genetically modified astrocyte of claim 65 wherein said regulatable promoter comprises a constitutive promoter.

77. (New) The genetically modified astrocyte of claim 65 wherein said regulatable promoter comprises an astrocyte-specific promoter.

78. (New) A method of expressing DNA encoding a biologically active molecule in a subject which method comprises: obtaining a sample of the astrocyte of claim 65 comprising said DNA encoding said biologically active molecule; transplanting said astrocyte into said subject; and expressing said biologically active molecule in said astrocyte in said subject.

79. (New) The genetically modified astrocyte of claim 65 which additionally comprises a third DNA encoding a poison pill and wherein expression of the DNA encoding the poison pill is regulated by a regulatory element for controlling expression of said DNA.

REMARKS

The subject application is a continuation of U.S. Serial No. 08/862,438, filed May 24, 1997, now U.S. Patent No. 6,106,827 to issue on August 22, 2000, which is a continuation of U.S. Serial No. 07/909,281, filed July 6, 1992, now abandoned. A Notice of Allowance and Issue Fee Due was issued on March 29, 2000 in connection with Serial No. 08/862,438. On June 29, 2000, applicants paid the issue fee. Accordingly, U.S. Serial No. 08/862,438 is pending today and the subject application is co-pending therewith for the purposes of 35 U.S.C. §120.

By this Preliminary Amendment, applicants have amended the specification to provide an updated history of the parentage of

prior related applications. Applicants have also canceled claims 1-64 without disclaimer or prejudice and have added new claims 65-79. Accordingly, claims 65-79 are presently under examination.

Support for all the new claims may be found throughout the specification and claims as filed, as described below.

Support for new claim 65 may be found in claim 1 as filed, *inter alia*. New claim 65 is identical to claim 1 as allowed in parent application U.S. Serial No. 08/862,438, except that the limitations as to the specific promoter regulating expression of DNA encoding the selectable marker have been removed.

Support for new claims 66-74 may be found in claims 2, 3, 5, 8-10, 12, 13 and 17 as filed, *inter alia*. These new claims, dependent on claim 65, are identical to dependent claims 2, 3, 5, 8-10, 12, 13 and 17 as allowed in parent application U.S. Serial No. 08/862,438, except that in the parent application they are dependent on claim 1.

Support for the phrase "wherein the promoter regulating expression of DNA encoding the selectable marker is the thymidine kinase promoter" in new claim 75 is supported *inter alia* by page 12, lines 14-15 of the specification which recites "pMCINeoPolyA in TE buffer (Stratagene, Inc)". The skilled artisan would know that plasmid pMCINeoPolyA carries a thymidine kinase (TK) promoter. As further support, applicants enclose, as Exhibit A, the package insert supplied by Stratagene with this plasmid. In Exhibit A, plasmid pMCINeoPolyA is depicted, and clearly shows the TK promoter.

Support for the phrase "constitutive promoter" in new claim 76 is found *inter alia* in claim 14 as filed. Additional support is found

on pages 21-24 and Figs. 8-12 of the specification; these pages and Figures all demonstrate that there is a basal constitutive level of promoter activity without addition of dopaminergic drugs such as dopamine and apomorphine. These dopaminergic drugs add additional activity above the basal constitutive activity.

Support for the phrase "astrocyte-specific promoter" in new claim 77 is found *inter alia* in claim 15 as filed.

Support for the phrase "method of expressing DNA encoding a biologically active molecule in a subject" in new claim 78 is found *inter alia* in claim 46 as filed.

Support for the phrase "poison pill" in new claim 79 is found *inter alia* in claims 1 and 53 as filed, and in pages 25-26 of the specification.

It should be noted that none of the prior art cited in the prosecution of U.S. Serial No. 08/862,438 discloses stably transfected non-virally genetically modified non-tumorous astrocytes, which harbor a selectable marker and which express a biologically active molecule. New claim 65 is novel and non-obvious over the prior art and applicant is certainly entitled to a claim of the scope of new claim 65. All the other claims pending are dependent on new claim 65, and applicants are therefore also entitled to these dependent claims.

Applicants maintain that this Preliminary Amendment does not introduce new matter. Accordingly, applicants respectfully request entry of this Preliminary Amendment.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided.

No fee, other than the enclosed $345.00 filing fee, is deemed necessary in connection with this Preliminary Amendment. If any additional fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,

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Dkt. 60435-A/JPW/MMM

METHOD OF PRODUCING GENETICALLY MODIFIED ASTROCYTES AND USES THEREOF

This invention was made with support under Grant No. RR05736 of the National Institutes of Health. Accordingly, the U.S. Government has certain rights in the invention.

Field of the Invention

This invention relates in general to gene therapy, and more particularly to gene therapy utilizing genetically modified astrocytes. The astrocytes are genetically modified using non-viral transfection methods, such as a calcium phosphate procedure. This enables a foreign gene of interest to be expressed by the modified astrocyte in a human patient or animal subject, thereby being useful for gene therapy in the central nervous system. In addition, this technology can be utilized for prevention of illness and modification of normal neuroendocrine function, and can be packaged as a kit.

Background of the Invention

Transplantation has become a major therapeutic option for a number of diseases over the past 20 years (Starzl et al., N Engl J Med 320:1014-1021,1092-1099 (1989); TINS 14(8):all pages (1991);

Murray, Science 256:1411-1416 (1992)]. In fact, transplantation of many portions of the central nervous system has been achieved in rodents and other species, including animal models of nigrostriatal dysfunction related to Parkinson disease [Lindvall et al., Science 247:574-577 (1990); Goetz et al., New Engl J Med 320:337-341 (1989); Gill and Lund, J Am Med Assoc 261:2674-2676 (1990)].

Gage et al., in U.S. Patent No. 5,082,670, issued January 21, 1992, discloses the use of genetically modified (by means of retrovirus insertion of genes) fibroblast donor cells for grafting into the central nervous system (CNS) to treat diseased or damaged cells. The fibroblast donor cells can be modified to produce a protein molecule capable of affecting the recovery of cells in the CNS. The entire contents of U.S. Patent No. 5,082,670 are hereby incorporated by reference into the subject application in order to more fully describe the state of the art of the subject invention.

Another cell which has been transplanted into the CNS is the astrocyte [Zhou et al., J Comp Neurol 292:320-330 (1990)]. Astrocytes have a wide range of functions, including: release of growth and trophic factors; inactivation of neurotransmitters; antigen presentation; ionic regulation; and response to certain lymphokines [Lillien and Raff, Neuron 5:111-1219 (1990); Raff, Science 243:1450-1455 (1989); Kimelberg and Norenberg, Scientific American, pp. 66-76 (April 1989)]. In addition, astrocytes from neonatal and adult sources (including human brain) replicate *in vitro*. Moreover, unlike fibroblasts, astrocytes belong in the brain and have region specific properties [Shinoda et al., Science 245:415-

417 (1989); Batter and Kessler, Molec Brain Res 11:65-69 (1991)]. When transplanted, astrocytes survive at the site of injection and may migrate up to several millimeters into the host brain without forming tumors [Zhou et al. (1990)]. Some of the potential advantages of using astrocytes over skin fibroblasts concern this migration into the host brain, as well as lower epileptogenicity [Jennett, Arch Neurol 30:396-398 (1974)], and their natural expression of neurotransmitter receptors. Furthermore, although inadvertently displaced normal (primary) fibroblasts following spinal taps form spinal fibroma and transplants of established neuronal cell lines (e.g. C6-glioma, PC12 cells, etc.) often form neoplastic tumors, this has not occurred with astrocyte transplantation [Zhou et al. (1990); Emmett et al., Brain Res 447:223-233 (1988)]. Indeed, astrocytes only migrate away with little if any new cell division. In contrast, fibroblasts do not migrate and are limited by a reactive gliosis surrounding the transplant [Kawaja et al., J Comp Neurol 307:695-706 (1991)] while astrocytes can interdigitate between neurons after migration and thus have direct contact with neurons [Zhou et al. (1990)].

In addition to the choice of a particular cell for transplantation, a method for modifying the particular cell must also be chosen. A common method, such as the method disclosed in Gage et al., is viral-mediated gene transfer. Viral-mediated gene transfer raises safety issue problems due to the use of active and potentially pathogenic viruses [Amer Soc for Microbio News 58(2):67-69 (1992)]. For example, the biological properties of retroviruses utilized by Gage et al. have potential for causing

mutations or cancer, and the possibility of continued infectivity. Furthermore, the physical dimensions of retroviruses limit the amount of foreign DNA which can be transferred via the retrovirus.

Another alternative method of gene transfer is chemical mediated gene transfer, such as by stable calcium phosphate transfection. The parameters for transfecting cells by this method vary for each different cell type, and therefore need to be determined and optimized for each different cell type.

Summary of the Invention

It is thus an object of the subject invention to provide genetically modified normal (primary) astrocytes which can be utilized in gene therapy. It is a further object to provide such genetically modified astrocytes utilizing a chemical transfection means such as calcium phosphate transfection.

It is also an object of this invention to provide plasmids and various vectors for transfecting such astrocytes.

Also provided are methods of utilizing the genetically modified astrocytes, selecting for them, inducing the gene of interest, and a "poison pill" method, etc.

In accordance with these objectives, the invention provides genetically modified normal (primary) astrocytes which can be maintained in selective media for over one year or can be released to rapidly expand the population in vitro after at least three weeks of selection (see below). In such astrocytes, a stably incorporated expressed gene can be readily detected in vitro prior to transplantation. These cells can be identified in

vivo following transplantation into the striatum for at least three weeks by Nissel staining, by GFAP staining, and by detection of the gene of interest (e.g. the reporter gene chloramphenicol acetyl transferase activity). Other methods of cell detection include PHAL lectins, microbeads, fluorescein dyes, and ^{3}H-Thymidine. Furthermore, the expression of a transfected promoter construct (pENKAT12) can be regulated by dopaminergic receptor pathways in such astrocytes.

Brief Description of the Figures

These and other objects, features and advantages of this invention will be evident from the following detailed description of preferred embodiments when read in conjunction with the accompanying drawings in which:

Figure 1 illustrates CAT activity for transfected astrocytes in the presence and absence of selective pressure in vitro;

Figure 2 illustrates CAT activity in vivo after transplant of stably transfected astrocytes;

Figure 3 illustrates the construction of plasmid pENKTH2;

Figure 4 illustrates the construction of plasmid pENKHTH1;

Figure 5 illustrates the construction of plasmid pENKBASIC;

Figure 6 illustrates the construction of plasmid pENKBASIC-B;

Figure 7 illustrates the construction of plasmid pGF8neo;

Figure 8 is a dose response curve for dopamine on the inducability of pENKAT12 in cultured rat astrocytes;

Figure 9 is a dose response curve for apomorphine on the inducability of pENKAT12 in cultured rat astrocytes;

Figure 10 is a dose response curve for SKF38393-R(+) (D1-receptor agonist) on the inducability of pENKAT12 in cultured rat astrocytes;

Figure 11 is a dose response curve for LY17155 (D2-receptor agonist) on the inducability of pENKAT12 in cultured rat astrocytes;

Figure 12 illustrates that dopaminergic receptor subtypes interact to regulate transfected primary rat astrocytes; and

Figure 13 illustrates that dopamine alone induces the endogenous rat ppEnk gene.

Detailed Description of the Invention

MATERIALS AND METHODS

Plasmid Constructions

All plasmids for use in development, prevention and therapeutic purposes were made using standard restriction enzyme modification, and other DNA isolation, preparation, and ligation as required. These standard methods are summarized by Ausubel et al., in Current Protocols in Molecular Biology, Wiley & Sons, New York, New York (1992), and by Sambrook et al., in Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Press, Cold Spring Harbor, New York (1989).

Site-specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these

commercially available restriction enzymes. (See, e.g. New England Biolabs, Product Catalog.) In general, about 1 μg of plasmid or DNA sequences is cleaved by one unit of enzyme in about 20 μl of buffer solution. Typically, an excess of restriction enzyme is used to insure complete digestion of the DNA substrate. Incubation times of about one hour to two hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether extraction,, and the nucleic acid is recovered from aqueous fractions by precipitation with ethanol. If desired, size separation of the cleaved fragments may be performed by polyacrylamide gel or agarose gel electrophoresis using standard techniques. A general description of size separations is found in Current Protocols in Molecular Biology (1992).

Restriction cleaved fragments may be blunt ended by treating with the large fragment of <u>Escherichia coli</u> DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using incubation times of about 15 to 25 minutes at 20°C to 25°C in 50 mM Tris (pH 7.6), 50 mM NaCl, 6 mM $MgCl_2$, 6 mM DTT and 5-10 μM dNTPs. The Klenow fragment fills in at 5' sticky ends but chews back protruding 3' single strands, even though the four dNTPs are present. A more efficient method of chewing back protruding 3' overhangs is by using T4 DNA polymerase instead of the Klenow fragment. After treatment with Klenow or T4 DNA polymerase, the mixture is extracted with phenol/chloroform and ethanol precipitated. Treatment under appropriate conditions with S1 nuclease or Bal-31 results in hydrolysis of any single-stranded portion.

Ligations are performed in 15-50 μl volumes under the following standard conditions and temperatures: 20 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33 mg/ml BSA, 10 mM-50 mM NaCl, and either 40 μM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt-end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 $\mu g/ml$ total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt end ligations (which can be performed employing a 5-30 fold molar excess of linkers) are performed at 1 μM total ends concentration.

In vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) or calf intestinal alkaline phosphatase (CIP) in order to remove the 5' phosphate and prevent religation of the vector. Digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of Na^+ and Mg^{+2} using about 1 unit of BAP or CIP per mg of vector at 55 to 60°C for about one hour. In order to recover the nucleic acid fragments, the preparation is extracted with phenol/chloroform and ethanol precipitated. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

Culturing of Rat Astrocytes:

Two day old Sprague Dawley rat pups were sacrificed by decapitation. After the skull was opened and the brain removed, it was placed in CMF-Sal G (calcium magnesium free P-SAL G) in a culture

dish on ice [Vilijn et al., Proc Natl Acad Sci USA 85:6551-6555 (1988)]. Striata from ten animals were microdissected to seed approximately 30 (1.5 ml) dishes at 5 x 10^5 cells per dish. This tissue was minced with forceps, transferred to a 15 ml sterile conical tube, and the supernatant that remained after momentary settling was used to rinse the culture plate. The tissue was then centrifuged (500-1000xg, 1 minute), the supernatant was aspirated off, and the cells were resuspended in 2 ml of 0.1% trypsin (1.0% Gibco #610-5095AE diluted 1:10 v/v with CMF-Sal G) and allowed to incubate for 30 minutes at 37°C. Incubation was followed by recentrifugation (500-1000xg, 1 minute) and resuspension of the pellet in 2 ml of complete media by gentle trituration until a uniform suspension was seen. The cells were plated at a density ratio of 5 x 10^5 cells per 1.5 ml of complete media (swirled gently) on poly-D-lysine (Sigma #P7886, pH 8.5) coated plates (35 mm dish, Falcon #3001) (1.0 X 10^6/10 ml for 100 mm dish, Falcon #3003) and incubated at 37°C, 100% relative humidity and 5% CO_2, for five to six days. The media was then replaced with ice cold media (1.5 ml for 35 mm dish; or 10 ml for 100 mm dish) and the dishes were agitated to remove neural non-adherent cells [Vilijn et al. (1988)]. Subsequently, the media (37°C) was changed every 4 to 5 days, until the cells grew to confluency (about two weeks), and then the cells were passaged every 3 weeks using trypsin (see below) to release the cells from the poly-D-lysine coated plates. At this point, the cells were either used for transfection or for primary culture experiments.

Identity of the astrocyte cells was validated by glial fibrillary acidic protein (GFAP) staining and morphology. Astrocytes at low density have star-like

shapes and are very flat; at high density they form a "cobble-stone" pattern. Neurons, on the contrary, have long processes (neurofilaments), and are less than 1% of the cells. Fibroblasts look very similar to astrocytes, but are GFAP negative. Oligodendrocytes are dark cells with short processes which are much smaller than astrocytes and sit on the surface of the astrocytes. Using the above-described protocol, over 95% of the astrocyte cells were GFAP positive.

Replating Protocol

Cells are replated by placing 2-3 ml of Serum Free Medium or PBS x 2 in each 100 mm plate and adding 0.05% Trypsin-EDTA, Gibco #610-5300Af [0.5 ml in 1.5 ml Dish (30 mm); 1.0 ml in 5 ml Dish (60 mm); 2.0 ml in 10 ml Dish (100 mm)]. Incubate at 37°C for 5 minutes, then tap culture dish 25 times to release rounded up cells. Pool samples and add 1:1 (v/v) media with serum. Centrifuge for 5 minutes at 1000 rpm (500-1000g). At this point, consider repeating trypsin treatment of the original plates. Then resuspend the cells in an appropriate volume and count an aliquot. Replate at about 0.5 x 10^6/30 mm Dish, 1.0 x 10^6/60 mm Dish, or 2.0 x 10^6/100 mm Dish (or one-half this amount for transfection).

Cell Handling After Transfection: Near confluent astrocyte cultures were replated at 1 X 10^6 cells per 100 mm culture dish, and then plasmids (pRSVCAT or pENKAT12, 10 μg) were introduced into astrocytes by the calcium phosphate transfection procedure. Stably transfected cells (see next section) were developed by co-transfection of 10-15 μg of a promoter reporter ("gene of interest") and 3 μg of pMCINeo PolyA

(Stratagene) (or equivalently pRSVNEO) followed by glycerol shock 6-7 hours later. Then the media covering the cells was changed to selective media 16-18 hours later. The cells were then maintained for at least 3 weeks in selective medium containing G418 (300 μg/ml; note - 100% mortality of cells which do not contain a resistance gene occurs at less than 200 μg/ml G418 within 14 days). G418-resistant astrocytes were grown in culture for at least 3 additional weeks without selective pressure prior to transplantation. A portion of stably transfected cells were harvested and lysates assayed for CAT enzyme activity [Gorman et al., Molecular Cellular Biology 2:1044-1051 (1982)]. Remaining cells were used for transplantation.

Following transfection of primary astrocytes with pRSVCAT, approximately 5% of cells were immunoreactive to the CAT protein with variable intensity of staining prior to selective pressure (e.g. after 24-48 hours). After selective pressure was applied, CAT positive cells are seen. At this stage 100% of cells are of this phenotype.

Figure 1 illustrates CAT bioactivity during and after the release of selective pressure in vitro. Astrocytes were transfected, maintained in selective medium for 3 weeks, and released from selective pressure for 3 more weeks. Transfected astrocytes were harvested at the time points indicated. The marked rise in CAT activity at 42 days was associated with a dramatic rise in the number of astrocyte cells per dish in the absence of selective pressure. Stably transfected astrocytes have been maintained in culture with selective pressure for over one year. These results indicate that stably transfected astrocytes can maintain expression of the RSVCAT gene

product for at least 3 weeks in vitro without selective pressure and can be maintained in culture for at least one year with selective pressure. This situation is similar to the absence of selective pressure that exists in vivo after short term transplantation.

Calcium Phosphate Transfection Protocol

Add DNA sequentially to 1 ml HeBS buffer [137 mM NaCl; 5 mM KCl; 0.7 mM Na_2HPO_4; 6 mM dextrose; 21 mM HEBS (pH 7.1)] in snap cap sterile polypropylene tubes (12 x 75 mm; Falcon #2063). For stables (ratio 4/1 or 5/1), add 15 μg of test plasmid in TE Buffer, then add 3.0 μg pMCINeo PolyA in TE Buffer (Stratgene, Inc.)(or pRSVNeo) and mix. For transients, use 10-15 μg of plasmid.

Then add 62.5 μl of 2M $CaCl_2$ and wait 30 minutes or less to allow fine crystals to form (tiny dots will be seen under a microscope, not clumps; excess time results in larger crystals which are less efficient in getting into the cells). During the crystal forming stage, wash culture plates with media minus serum two times (e.g. 1/2 vol of dish or about 5 ml) and aspirate to nearly dry. Note that plates were seeded on the previous day with 10^6 cells per 10 ml dish.

At 30 minutes, add 1.062 ml $CaPO_4$/DNA precipitate mix to the center of the plate on a level surface (avoid bubbles on the plate), and wait 30 minutes (swirl every 10 minutes to keep monolayer wet) at about 37°C for astrocytes. After 30 minutes, gently add 10 ml of complete media dropwise to slow stream to avoid dislodging cells.

At this point, wait 6 to 7 hours, then remove media until nearly dry. Glycerol shock cells by

adding 2 ml of HeBS Buffer (15% glycerol) per dish for 90 seconds (should kill approximately 75% of cells). Then aspirate off and wash by adding media minus serum (dropwise, e.g. 5 ml for 10 ml plate or 1/2 volume of plate); rotate plate to rinse corners. Aspirate media off again, and then add 10 ml of complete media (dropwise, gently) to the center of the plate. The following day add the G418 antibiotic (12-18 hours may be best) at a G418 final concentration of 300 μg/ml (final) in HEPES. For example, add 100 μl per 10 ml of 30 mg/ml G418 solution. To facilitate regrowth, release selection after 3 weeks (e.g. no more G418). Prior to release change media every 4-5 days. Replate when the cells are 90% confluent.

Transplant Protocol: All surgical procedures are performed aseptically under equithesin anesthesia (a mixture of chloral hydrate and sodium pentobarbitol at 50/50 v/v), after placement of a small burr hole. Recipient rats received a 5 μl injection of 30,000 to 500,000 cells in PBS with or without 33 mM glucose injected through a 10 μl Hamilton microsyringe (18 or 25 Gauge needle). The needle is positioned stereotaxically into the left or right striatum and each injection is made over 3 minutes. Following injections, the needle was left in place for 1 minute before slow withdrawal. Sham grafts (negative controls) consisted of an equal volume of saline or untransfected astrocytes injected in the same manner.

CAT Assay: Tissue is harvested for assay of CAT enzyme activity by dissecting the brain region with the transplant (tissue block of 2 x 2 x 4 mm around transplant, a border of about 1-2 mm, approximately

50 mg tissue). Freeze on dry ice and pulverize in porcelin mortar on liquid nitrogen. Rinse fragments into Eppendorf with liquid nitrogen allowing it to evaporate on dry ice. Add 70 μl of 0.25 M Tris (pH 7.8) and cycle to 37°C then -70°C three times. Recover a 50 μl supernate aliquot (after centrifuging) into a clean tube. Then mix sequentially 34 μl ddH_2O, 70 μl 1 M Tris (pH 7.8), 25 μl extract, and 1 μl of C^{14}-chloramphenicol (0.1 μCi/tube). Pre-incubate tubes at 37°C for 5 minutes. Then add 20 μl Acetyl CoA (4 mM, lithium salt) and incubate for 60 minutes at 37°C. Extract with 1 ml ethyl acetate by collecting upper organic layer (vortex 30 seconds, microcentrifuge 30 seconds). Dry, then resuspend in 25 μl ethyl acetate, spot and separate on TLC (thin layer chromatography) plates (Chromagram #13179, Eastman Kodak - no fluorescence) in 95/5 v/v chloroform/methanol for two hours. Dry plates, coat with C14 enhancer (e.g. with Resolution by EM Corp.), allow to dry, and then expose autoradiograph for 2 days or longer (at -80°C with fluorescent screen) before analyzing by densitometer for quantitation, or scintilation counting for quantitation.

Figure 2 provides evidence that the CAT gene is expressed in the brain after transplant of stably selected transfected astrocytes. CAT activity was detected 3 weeks after transplantation of stably transfected astrocytes in the appropriate hemisphere. CAT enzyme activity was not affected by the presence of brain tissue in the extract.

<u>Histology</u>: Rats were perfused transcardially under deep equithesin anesthesia with 4% paraformaldahyde in 0.1 M phosphate buffer. Fixation was continued

for 2-24 hours, followed by cryoprotection in graded 10-30% sucrose in the same buffer, freezing on dry ice, and cryostat sectioning at 30 μm. Coverslips were fixed in the same solution for 10 minutes or methanol:acetone 1:1 for 2 minutes. Freefloating sections and coverslip were washed in 0.1M phosphate buffered saline pH 7.2-7.4 (PBS), treated with 0.2% TritonX-100 for 30 minutes. Primary antibodies were rabbit anti-chloramphenicol acetyltransferase (CAT) antibody, 1:10 to 1:20,000 (5 Prime-3 Prime, Inc., Boulder, Colorado), Histogen GFAP monoclonal antibody (Biogenex Labs, San Ramon, California) and beta-Gal antibody, 1:500 to 1:2,000. Each was diluted in PBS containing 3% goat serum and 0.3% TritonX-100. Antibody binding was visualized with Vectastain ABC (Vector Labs, Burlingame, California) and diaminobenzidine. Control sections were reacted with the primary antibody omitted or replaced with an unrelated antibody. Adjacent sections were mounted serially and stained with cresyl violet.

Transient Transfection of Astrocytes For Rapid Drug Assay - Receptor Evaluation

Following transient transfection with plasmid pENKAT12 [Comb et al. (1986)] without a Neo gene plasmid [Graham and Van der Eb, Virology 53:456-457 (1973); Weisinger et al., Oncogene 3:635-646 (1988)], astrocytes were treated with drugs (see below). On harvest, the cell lysates were assayed for CAT expression (the transfected reporter gene, a bacterial gene not present in eukaryotes) [Gorman et al. (1982); Weisinger et al. (1988)]. Transfection efficiencies were standardized by Southern analysis of plasmid DNA in Hirt lysates [Hirt, J Mol Biol 26:365-369 (1967); Weisinger et al. (1988)].

To quantitate CAT activity, 20 μl of each cell lysate was used to acetylate [^{14}C]chloramphenicol [Lopata et al., Nuc Acids Res 12:5707-5717 (1984); Weisinger et al. (1988)] (see protocol above). Chloramphenicol and its acetylated derivatives were separated by ascending silica gel thin layer chromatography ($CHCl_3$:CH_3OH, 95:5 v:v), visualized by autoradiography [Weisinger et al. (1988)], and analyzed with a densitometer (see above details) or by scintillation counting of TLC spots.

For RNA analysis, total RNA was prepared by the acid guanidinium thiocyanate/phenol/chloroform method of Chomczynski and Sacchi [Chomczynski and Sacchi, Anal Biochem 162:156-159 (1987)], as modified [Weisinger et al., J Biol Chem 265:17389-17392 (1990); LaGamma et al. Molec Br Res 13:189-197 (1992)]. Total RNA was quantified by optical density and 10 μg aliquots were fractionated on 1% glyoxal gels and transferred to Nytran (S&S) or nylon Biotrans (ICN) membranes. Northern blot prehybridization and hybridization solutions were as previously described [LaGamma et al. 1992]. Briefly, each RNA blot was hybridized at 45°C to a radiolabelled double stranded coding region fragment of ppEnk cDNA (pRPE2) or glyceraldehyde-3-phosphate dehydrogenase (pRGAPDH-13) for 24-48 hours. A PvuII digest of plasmid pRPE2 [Yoshikawa et al., J Biol Chem 259:14301-14308 (1984)] yielded a 435 bp exon 3 fragment, which was labelled with ^{32}P-dCTP using random primer labelling kits (Prime-it; Stratagene). Blots were rehybridized to a PstI 1,085 bp fragment of pRGAPDH-13 [Piechaczyk et al., Nuc Acids Res 12:6951-6963 (1984)] as an RNA loading control. Following each hybridization, the blots were washed

at 60°C in 0.2X SSC/0.1% SDS for 30 minutes and again at 50°C and then autoradiographed.

Evaluation of drug treatments were performed after plasmid pENKAT12 [Comb et al. (1986)] was introduced into the cells. The day after the transient transfection, the cultures were treated with either dopaminergic or serotonergic drugs at various concentrations for a further 16-18 hours. Following drug treatment the cultures were then harvested, and cell extracts were made and assayed for both chloramphenicol acetyl transferase (CAT) activity and levels of transfected plasmid (Hirt lysates) as discussed above, or for endogenous RNA levels.

All drugs were made up in sterile PBS and then resterilized through Acrodisc13 (0.2 μm; GelmanSciences) and added to each 1.5 ml culture in a final volume of 0.1 ml. Dopamine-HCl, Apomorphine-HCl, SKF38393-R(+), Ly17155, SCH39166, s(-)-Sulpiride, Serotonin-HCl, 5-methoxytryptamine and Buspirone were purchased from Research Biochemicals Inc. (Massachusetts). In the combined drug experiments both drugs were added simultaneously and maintained for the entire 16-18 hours. Following harvesting and extraction, CAT assays were run (see above).

Autoradiograms were quantified by two dimensional scanning densitometry using a LKB 2400 Gelscan XL (Bromma, Sweden). Digitized data were analyzed with LKB Gelscan software (version 1.0) on an IBM AT computer, as previously described [Weisinger et al. (1990)]. Multiple autoradiogram exposures of the same experiments were analyzed so that band or spot intensities reported represented sub-saturation values. One-way analysis of variance

was performed on the data, followed by Newman-Keuls test, where appropriate [Zar, in Biostatistical Analysis, pp. 101-162, Prentice-Hall, New Jersey (1974)].

EXAMPLE 1

Construction of Plasmid pENKTH2

Referring to Figure 3, plasmid pENKAT12 (Comb et al. 1986) was restricted using HincII followed by NcoI. This linearized plasmid was then treated with bacterial alkaline phosphatase (BAP) twice, in order to remove the 5' phosphate and prevent future religation of the vector on itself. A 1900 base pair BamHI-HindIII DNA fragment containing the rat tyrosine hydroxylase from the prTH122 plasmid (supplied by Dr. K. O'Malley, Washington University, St. Louis, MO) after having its 5' overhangs flushed using the Klenow fragment of *Escherichia coli* polymerase, was ligated into the HincII backbone of the above linearized pENKAT12. pENKTH2 was the resultant form that allowed sense rat tyrosine hydroxylase transcription from the human preproenkephalin gene promoter.

Application of Plasmid pENKTH2

This vector will allow expression of the tyrosine hydroxylase gene product in astrocytes for use in animal models of Parkinson's disease or in human therapy for Parkinson's disease, where increased activity of this tyrosine hydroxylase enzyme can produce dopamine and alleviate functional deficits.

EXAMPLE 2

Construction of Plasmid pENKHTH1

Referring to Figure 4, a 1784 base pair EcoRI fragment derived from pMV-7 [Horellou et al., Proc Natl Acad Sci USA 86:7233-7237 (1989)], containing the human tyrosine hydroxylase gene (HindIII-BstXI fragment) was isolated and had its EcoRI 5' overhangs flushed using the Klenow fragment of *Escherichia coli* polymerase. This fragment was then ligated into the HincII backbone of the above linearized pENKAT12. The correctly oriented form of this plasmid was selected such that sense transcription of the human tyrosine hydroxylase gene was generated following RNA initiation at the human preproenkephalin promoter. This plasmid was designated pENKHTH1.

Application of Plasmid pENKTH1

This vector differs from pENKTH2 only in that the human tyrosine hydroxylase (TH) gene is expressed. The usefulness of TH expression in Parkinson's therapy is similar to that discussed for plasmid pENKTH2 above.

EXAMPLE 3

Construction of Plasmids pENKBASIC and pENKBASIC-B

Plasmids pENKBASIC and pENKBASIC-B had double stranded synthetic custom polylinkers with HincII ends ligated into the same HincII restricted, BAP treated pENKAT12 backbone used in the previous two constructs. Both polylinkers had 11 unique 6mer or better unique restriction enzyme recognition sites between two HincII sites. The pENKBASIC polylinker had the following set of restriction sites: HincII, KpnI, HpaI, BclI, XhoI, ClaI, StuI, BglII, NotI, XmaIII, SacII, BstXI, HincII. The pENKBASIC-B polylinker has the following set of restriction

sites: HincII, KpnI, HpaI, BclI, XhoI, SmaI/ApaI, PstI, BglII, NotI, PvuI, SacI, SphI, HincII. Each vector is designated with a "+" or "-" depended on the orientation of the polylinker, with respect to the preproenkephalin promoter (see Figures 5 and 6).

Application of Plasmids pENKBASIC and pENKBASIC-B

These generic vectors will allow any gene of interest to be expressed and regulated by the human enkephalin promoter. The polylinkers facilitate the insertion of any coding region sequence into the splice site.

EXAMPLE 4

Construction of Plasmid pGF8neo

Referring to Figure 7, the plasmid pSV_2neo (commercially available from the ATCC - American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 U.S.A.) was restricted with AccI and treated twice with BAP. AccI-HindIII adaptor fragments were ligated into the above linearized pSV_2neo to make pSV_2Hneo. This plasmid was then further restricted with HindIII and again treated twice with BAP. Into this linearized plasmid a 268 base pair GFAP promoter containing HindIII fragment was ligated. This GFAP fragment was HindIII restricted from the plasmid pGF8L [Miura et al., J Neurochem 55:1180-1188 (1990)]. Only the plasmid with the GFAP promoter driving a sense neo gene was designated pGF8neo.

Application of Plasmid pGF8neo

For an application of plasmid pGF8neo, see details below concerning the "poison pill".

EXAMPLE 5

The effects of dopaminergic and serotonergic receptor agonists and antagonists in cultures of primary rat astrocytes were examined. Astrocytes were transiently transfected with a chimeric human preproenkephalin promoter (human ppEnk)-bacterial chloramphenicol acetyl transferase plasmid (pENKAT12 of Comb et al. [Comb et al., Nature 323:353-356 (1986)] and treated with different dopaminergic and serotonergic drugs. The resulting agonist induced effects were compared to the effects on the endogenous rat ppEnk gene (under control of the endogenous rat ppEnk promoter) in replicate cultures. The dopaminergic agonists were found to induce a response in the transfected pENKAT12 plasmid while serotonergic agonists did not. Furthermore, while there was a dopaminergic induction of expression of the transfected gene under control of the human ppEnk promoter, there was only a marginal effect on the induction of the endogenous rat ppEnk promoter.

Dose response curves for the effect of dopaminergic agonists on the inducability of pENKAT12 in cultured rat astrocytes was generated using the above methods, as shown in Figures 8-11. Dopamine and apomorphine have both D1 and D2 receptor agonist activities [Kebabian and Calne, Nature 277:93-96 (1979)] and they both induce episomal pENKAT12 plasmid expression (under control of the human ppEnk promoter) about 19 fold when present at 10^{-5} Molar (Figures 8 and 9). SKF38393-R(+) (Figure 10) is a D1 agonist and LY17155 (Figure 11) is a D2 agonist.

Additionally, the responsiveness of the transfected cultures to serotonergic (5HT) agonists was assessed. Cultured primary astrocytes have been reported to have functional 5HT receptors [Hertz et

al., Can J Physiol Pharmacol 57:223-226 (1979); Hosli and Hosli, Neurosci Lett. 65:177-182 (1986); Hansson, Progr in Neurobiol 30:369-397 (1988); Whitaker-Azmitia et al., Brain Res 528:155-158 (1990)] that can be induced to increase c-AMP levels in these glial cells [Hertz et al. (1979); Hosli and Hosli, J Physiol 82:191-195 (1987); Hansson et al., Neurochem Res 9:679-689 (1984); Whitaker-Azmitia, in Glial Cell Receptors, pp. 107-120, ed. Kimelberg, Raven Press, New York (1988)]. Astrocytes were treated with either of three serotonergic agonists, serotonin, 5-methoxytryptamine and buspirone, at the same concentration as the dopaminergic agonists.

Serotonergic agonist treatments showed no significant changes in transfected CAT expression. In these studies, dopamine (10 μM) treatments of transfected astrocyte cultures were performed in parallel as positive controls.

Figure 12 illustrates that the dopaminergic receptor subtypes interact to regulate transfected primary rat astrocytes. Dopamine alone induced the ppEnk gene and its effects are blocked by appropriate agents. Groups of 6 to 9 dishes were analyzed and data reported as X +/- SEM. Comparisons were made by ANOVA followed by Neuman-Keuls test: * $p < 0.005$ vs all other groups; ** $p < 0.02$ vs all other groups except D1 agonist, D2 agonist, and D1 + D2 agonist groups; + $p < 0.001$ from dopamine alone as are the vehicles and both blockers alone. All drugs were used at 10 μM for 16 hours. D1 Agonist is SKF38393-R(+); D1 Blocker is SCH39166; D2 Agonist is LY17155; and D2 Blocker is S(-)-Sulpiride.

Regulation of the Endogenous ppENK gene:
Promoter Comparison

To determine whether the signal transduction pathway involved with the induction of the transfected human ppEnk promoter is relevant to the regulation of the endogenous rat ppEnk gene, northern blot analysis was performed in parallel experiments. The northern data showed that the endogenous rat ppEnk promoter was only marginally induced 2.7 fold (compared to the transfected human exogenous ppEnk promoter) by dopamine (10 μM) (Figure 13, p=0.05) over the untreated control. This indicates the predominant effect of drug treatment is on the transfected gene.

This highlights a difference between the transfected human ppEnk promoter versus the endogenous rat ppEnk promoter in the same cell background after similar treatments.

These results demonstrate that the human ppEnk promoter transfected into "normal" primary striatal astrocytes can be induced with dopaminergic agonists. Based on these results, one concludes that L-DOPA, MAO inhibitors, or cholinergic pathway modifiers could be used to induce an engineered ppEnk promoter driven gene of interest (e.g. growth hormones or tyrosine hydroxylase gene) and to control local synthesis of the transfected gene product by dopaminergic pathways. Benefits like this are not currently available from other inducible promoters like the metallothionein [Hamer and Walling, J Mol Appl Genet <u>1</u>:273-288 (1982)] or the Mouse Mammary Tumor Virus (MMTV) [Yamamoto, in Molecular Developmental Biology: Expressing Foreign Genes, pp. 131-148, ed. Bogorad and Adelman, Alan Liss, New York (1985)] promoters, as the former promoter is induced

by heavy metals and the latter by high dose glucocorticoid hormones. The induction of both of these latter promoters in animals would involve toxic treatments or hormonal side effects and hence may not be useful in man. No other inducible promoters have been reported as functional in cells transplanted into the CNS.

In vivo Regulation of the human ppEnk promoter by Dopaminergic Pathways

To determine the extend of dopaminergic influence on basal levels of ppEnk promoter driven CAT activity, animals were unilaterally lesioned with 6-OHD injections into the Substantia Nigra. After establishing abnormal rotational behavior in these rats (Ungerstadt model of Parkinson's Disease), transiently transfected astrocytes (16-18 hours following transfection) were transplanted (500,000 cells/site) into the lesioned or contralateral striatum. Animals were treated with the combined dopaminergic agonist Apomorphine (0.3 mg/kg, ip, QID X4 doses), for 24 hours after transplantation and then sacrificed. The excised transplant-containing tissue blocks were assayed for CAT activity. ppEnk driven CAT activity was significantly ($p<0.05$) lower in all lesioned striata and was further reduced by apomorphine treatment ($p<0.05$). These data confirm the role of basal levels of dopaminergic input in maintaining high levels of expression of the transfected gene in the inervated striatum (see Figure 2). The apomorphine experiments indicate a pharmacologically induced down regulation of the ppEnk promoter, in vivo, therefore demonstrating control of an inserted gene in transplanted primary cells.

Poison Pill - Herpesvirus Thymidine Kinase

Principle advantages of astrocytes over other cell vehicles are their migratory capacity after transplantation, their regional specificity, and an ability to divide in culture (in vitro). As a result of these properties, and as a safeguard against the possibility of the transplanted cells growing out of hand during in vivo therapy, the invention provides a "poison pill" strategy which will render only transplanted cells susceptible to a pharmacologic agent. Cells modified (for example, using the above methods) to contain the herpes simplex thymidine kinase (HS-TK) gene become sensitive to treatment with the FDA-approved antiviral drugs gancyclovir and acyclovir [Moolten, Cancer Res 46:5276 (1986); Borrelli et al., Proc Natl Acad Sci USA 85:7572 (1988); Moolten and Wells, J Natl Cancer Inst 82:297 (1990); Ezzeddine et al., Neu Biol 3:608 (1991)]. Alternate methods for destroying unwanted transplanted cells would include genetically modifying astrocytes to express the bacterial enzyme cytosine deaminase which converts the generally non-toxic FDA-approved compound 5-fluorocytosine into the toxic product 5-fluorouracil, that will kill the genetically modified cells only [Mullen et al., Proc Natl Acad Sci USA 89:33 (1992)]. This can be most readily accomplished using the methodology of the subject invention by creating a plasmid vector containing a constitutive promoter (e.g. thymidine kinase or RSV as done with the CAT gene) driving a HS-TK reporter/product on the same sequence as the astrocyte-specific promoter GFAP driving a neomycin (G418) selection gene.

The G418 gene allows selective pressure in vitro and the TK poison pill gene allows selective

destruction with drugs in vivo. Neither of these approaches will alter the effects of the preceding sections where genetically modified astrocytes express other biologically active compounds. A simpler version of an astrocyte-specific selective pressure plasmid is illustrated in Figure 7 (pGF8neo).

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the following claims.

What is Claimed is:

1. A genetically modified astrocyte for gene therapy, said genetically modified astrocyte comprising:

one or more DNA sequences selected from the group consisting of DNA encoding a selectable marker, DNA encoding a poison pill, and DNA encoding a molecule useful for gene therapy; and

suitable regulatory elements for controlling expression of said one or more DNA sequences.

2. The genetically modified astrocyte of claim 1 wherein said selectable marker comprises neomycin resistance.

3. The genetically modified astrocyte of claim 1 wherein said selectable marker comprises methotrexate resistance.

4. The genetically modified astrocyte of claim 1 wherein said poison pill comprises herpes virus thymidine kinase.

5. The genetically modified astrocyte of claim 1 wherein expression of said DNA encoding said molecule useful for gene therapy results in the production of a protein.

6. The genetically modified astrocyte of claim 1 wherein expression of said DNA encoding said molecule useful for gene therapy results in the production of anti-sense RNA.

7. The genetically modified astrocyte of claim 1 wherein expression of said DNA encoding said molecule useful for gene therapy results in the production of a ribozyme.

8. The genetically modified astrocyte of claim 5 wherein said protein comprises a growth factor.

9. The genetically modified astrocyte of claim 8 wherein said growth factor comprises a cytokine.

10. The genetically modified astrocyte of claim 5 wherein said protein comprises tyrosine hydroxylase.

11. The genetically modified astrocyte of claim 1 wherein said suitable regulatory elements include a regulatable promoter.

12. The genetically modified astrocyte of claim 11 wherein said regulatable promoter comprises an inducible promoter.

13. The genetically modified astrocyte of claim 12 wherein said inducible promoter comprises a human preproenkephalin promoter.

14. The genetically modified astrocyte of claim 11 wherein said regulatable promoter comprises a constitutive promoter.

15. The genetically modified astrocyte of claim 1 wherein said suitable regulatory elements include an astrocyte-specific promoter.

16. The genetically modified astrocyte of claim 15 wherein said astrocyte-specific promoter comprises a promoter for glial fibrillary acidic protein.

17. An astrocyte cell line comprising the genetically modified astrocyte of claim 1.

18. A plasmid for transfection of astrocytes which plasmid comprises DNA encoding a molecule useful for gene therapy and suitable regulatory elements for controlling expression of said molecule useful for gene therapy.

19. A plasmid for transfection of astrocytes which plasmid comprises DNA encoding a selectable marker and suitable regulatory elements for controlling expression of said selectable marker.

20. The plasmid of claim 19 further comprising DNA encoding a poison pill and further suitable regulatory elements for controlling expression of said poison pill.

21. A plasmid for transfection of astrocytes which plasmid comprises DNA encoding a poison pill and suitable regulatory elements for controlling expression of said poison pill.

22. An astrocyte stably transfected with one or more plasmids, said one or more plasmids selected from the group consisting of:

a plasmid comprising DNA encoding a molecule useful for gene therapy and suitable regulatory elements for controlling expression of said molecule useful for gene therapy;

a plasmid comprising DNA encoding a selectable marker and suitable regulatory elements for controlling expression of said selectable marker;

a plasmid comprising DNA encoding a selectable marker and suitable regulatory elements for controlling expression of said selectable marker, and further comprising DNA encoding a poison pill and further suitable regulatory elements for controlling expression of said poison pill; and

a plasmid comprising DNA encoding a poison pill and suitable regulatory elements for controlling expression of said poison pill.

23. A method of stably transfecting primary cells, said method comprising stably transfecting said primary cells using non-viral transfection methods.

24. The method of claim 23 wherein said non-viral transfection method comprises chemical transfection.

25. The method of claim 24 wherein said chemical transfection comprises stable calcium phosphate transfection.

26. The method of claim 23 wherein said non-viral transfections method comprises electroporation.

27. The method of claim 23 wherein said primary cells comprise astrocytes.

28 A method for gene therapy in the central nervous system of a subject which method comprises:

genetically modifying primary cells to include DNA encoding a molecule useful for gene therapy in the central nervous system;

transplanting said genetically modified primary cells into the central nervous system of a subject; and

expressing said DNA encoding said molecule, thereby producing said molecule for gene therapy in the central nervous system of the subject.

29. The method of claim 28 wherein said primary cells comprise astrocytes.

30. The method of claim 29 wherein said astrocytes are genetically modified by a non-viral transfection method.

31. The method of claim 30 wherein said non-viral transfection method comprises chemical transfection.

32. The method of claim 31 wherein said chemical transfection comprises stable calcium phosphate transfection.

33. The method of claim 28 wherein said expression of said DNA is controlled by a regulatable promoter.

34. The method of claim 33 wherein said regulatable promoter is controlled pharmacologically.

35. The method of claim 34 wherein said pharmacologic control comprises utilizing dopaminergic pathways.

36. The method of claim 33 wherein said regulatable promoter comprises an inducible promoter.

37. The method of claim 33 wherein said regulatable promoter comprises a constitutive promoter.

38. A method of maintaining and growing astrocytes in culture, said method comprising:

growing first astrocytes with a liquid medium overlying said first astrocytes so as to condition said liquid medium;

removing said conditioned liquid medium; and

placing said removed conditioned liquid medium over second astrocytes, said removed conditioned liquid medium capable of maintaining and growing said second astrocytes in culture.

39. A method of selecting for astrocytes in a mixed cell population, said method comprising:

stably transfecting a mixed cell population with an astrocyte-specific plasmid, said astrocyte-specific plasmid comprising DNA encoding a selectable marker and suitable regulatory elements for controlling expression of said selectable marker;

growing said transfected mixed cell population under selective conditions, wherein said astrocyte-specific promoter functions only in transfected astrocytes present in said transfected mixed cell population, such that only transfected astrocytes present in said transfected mixed cell population can be selected under said selective conditions using said selectable marker under control of said astrocyte-specific promoter; and

selecting said astrocytes from said mixed cell population.

40. The method of claim 39 wherein said astrocyte-specific promoter comprises a promoter for glial fibrillary acidic protein.

41. The method of claim 39 wherein said selective marker comprises neomycin resistance.

42. The method of claim 39 wherein said selective marker comprises methotrexate resistance.

43. The method of claim 41 wherein said selective conditions include exposing said transfected mixed cell population to a neomycin analogue.

44. The method of claim 43 wherein said neomycin analogue comprises G418.

45. The method of claim 42 wherein said selective conditions include exposing said transfected mixed cell population to methotrexate.

46. A method of expressing a biologically active molecule in an astrocyte of a subject which method comprises:

obtaining a sample of an astrocyte;

stably inserting DNA encoding a biologically active molecule into DNA of said astrocyte;

transplanting said resulting astrocyte into a subject; and

expressing said biologically active molecule in said astrocyte in said subject.

47. The method of claim 46 wherein said biologically active molecule is selected from the group consisting of a protein, antisense RNA, and a ribozyme.

48. The method of claim 46 wherein said sample of an astrocyte is obtained by removing astrocytes from said subject.

49. The method of claim 46 wherein said stable insertion comprises a non-viral transfection method.

50. The method of claim 46 wherein said expression of said biologically active molecule is under control of a regulatable promoter.

51. The method of claim 50 wherein said regulatable promoter comprises an inducible promoter.

52. The method of claim 50 wherein said regulatable promoter comprises a constitutive promoter.

53. A method of killing astrocytes in a subject, said method comprising:

obtaining a sample of astrocytes;

stably transfecting said astrocytes with a plasmid, said plasmid comprising DNA encoding a poison pill and suitable regulatory elements for controlling expression of said poison pill;

transplanting said transfected astrocytes into a subject; and

exposing said transplanted transfected astrocytes to a selective condition, wherein said suitable regulatory elements cause expression of said

DNA encoding said poison pill only in said transplanted transfected astrocytes present in said subject such that only said transplanted transfected astrocytes present in said subject are killed by said selective condition due to said expression of said DNA encoding said poison pill under control of said astrocyte-specific promoter.

54. The method of claim 53 wherein said poison pill comprises herpse virus thymidine kinase.

55. The method of claim 54 wherein said exposure to a selective condition comprises exposure to a drug selected from the group consisting of acyclovir and gancyclovir.

56. A method of preventing deterioration of phenotypically normal cells in a subject which comprises:

detecting a genotype indicative of an eventual phenotypic abnormality in said normal cells;

treating said normal cell with the genetically modified astrocyte of claim 1 so as to prevent said phenotypic abnormality, said prevention being by expression of said DNA encoding said molecule useful for gene therapy by said genetically modified astrocyte.

57. The method of claim 56 wherein said phenotypic abnormality is indicative of Huntingtons disease.

58. An astrocyte maintained and grown by the method of claim 38.

59. An astrocyte selected by the method of claim 39.

60. A kit for gene therapy comprising the genetically modified astrocyte of claim 1.

61. A kit for gene therapy comprising the genetically modified astrocyte of claim 17.

62. A kit for gene therapy comprising one or more plasmids, said one or more plasmids selected from the group consisting of:

a plasmid comprising DNA encoding a molecule useful for gene therapy and suitable regulatory elements for controlling expression of said molecule useful for gene therapy;

a plasmid comprising DNA encoding a selectable marker and suitable regulatory elements for controlling expression of said selectable marker;

a plasmid comprising DNA encoding a selectable marker and suitable regulatory elements for controlling expression of said selectable marker, and further comprising DNA encoding a poison pill and further suitable regulatory elements for controlling expression of said poison pill; and

a plasmid comprising DNA encoding a poison pill and suitable regulatory elements for controlling expression of said poison pill.

63. The kit of claim 62 further comprising astrocytes to be transfected with said one or more plasmids.

64. A kit for gene therapy comprising:

a plasmid vector having a polylinker site for insertion of DNA encoding a gene of interest;

restriction enzymes for inserting said DNA at said site; and

the astrocyte of claim 58 to be transfected by the plasmid vector after insertion of said DNA into said plasmid vector.

METHOD OF PRODUCING GENETICALLY MODIFIED ASTROCYTES AND USES THEREOF

Abstract of the Disclosure

A genetically modified astrocyte for gene therapy is provided. The genetically modified astrocyte includes one or more stably introduced DNA sequences selected from DNA encoding a selectable marker, DNA encoding a poison pill, and DNA encoding a molecule useful for gene therapy. The genetically modified astrocyte may be produced utilizing plasmids and non-viral transfection methods, as are also provided by the subject invention. Methods for producing and utilizing the genetically modified astrocytes and regulating the engineered products, as well as kits thereof, are further provided.

FIG. 2

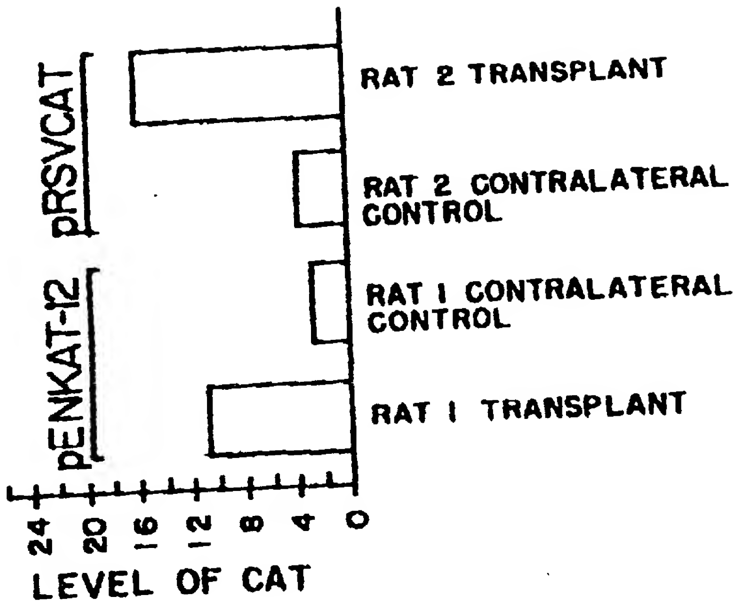

pENKAT-12
pRSVCAT
RAT 2 TRANSPLANT
RAT 2 CONTRALATERAL CONTROL
RAT 1 CONTRALATERAL CONTROL
RAT 1 TRANSPLANT
24
20
16
12
8
4
0
LEVEL OF CAT

FIG. 1

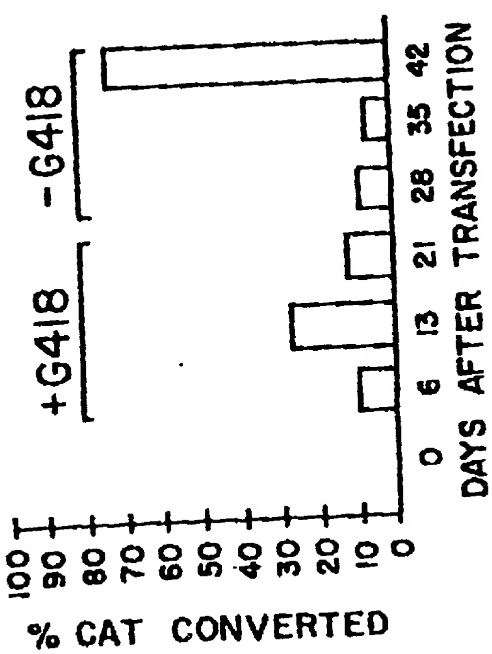
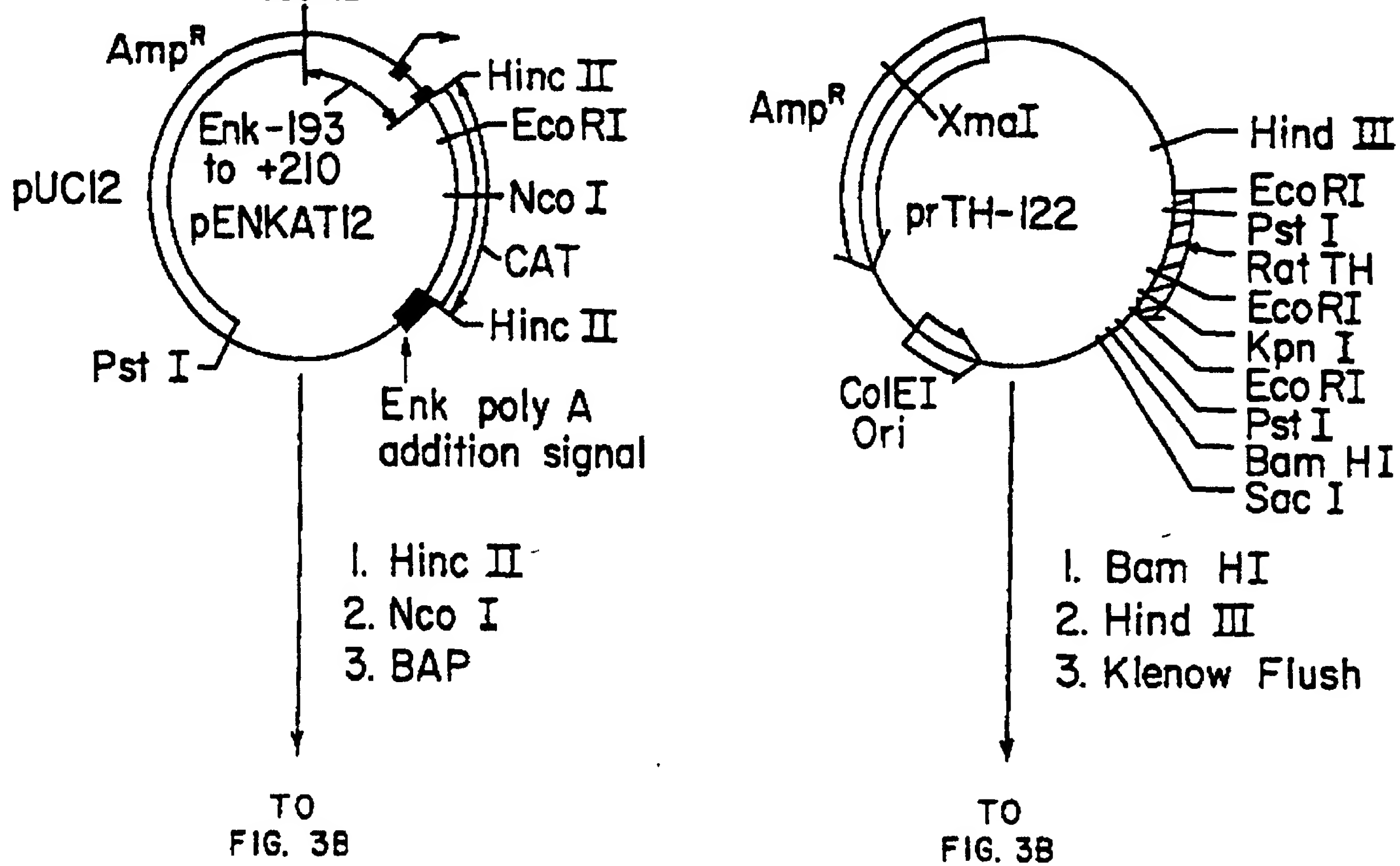

+G418
-G418
0
6
13
21
28
35
42
DAYS AFTER TRANSFECTION
100
90
80
70
60
50
40
30
20
10
0
% CAT CONVERTED

FIG. 3A


EcoRI
Amp^R
pUC12
Enk-193
to +210
pENKAT12
Hinc II
EcoRI
Nco I
CAT
Hinc II
Pst I
Enk poly A
addition signal
1. Hinc II
2. Nco I
3. BAP
TO
FIG. 3B
Amp^R
XmaI
prTH-122
Hind III
EcoRI
Pst I
Rat TH
EcoRI
Kpn I
EcoRI
Pst I
Bam HI
Sac I
ColEI
Ori
1. Bam HI
2. Hind III
3. Klenow Flush
TO
FIG. 3B

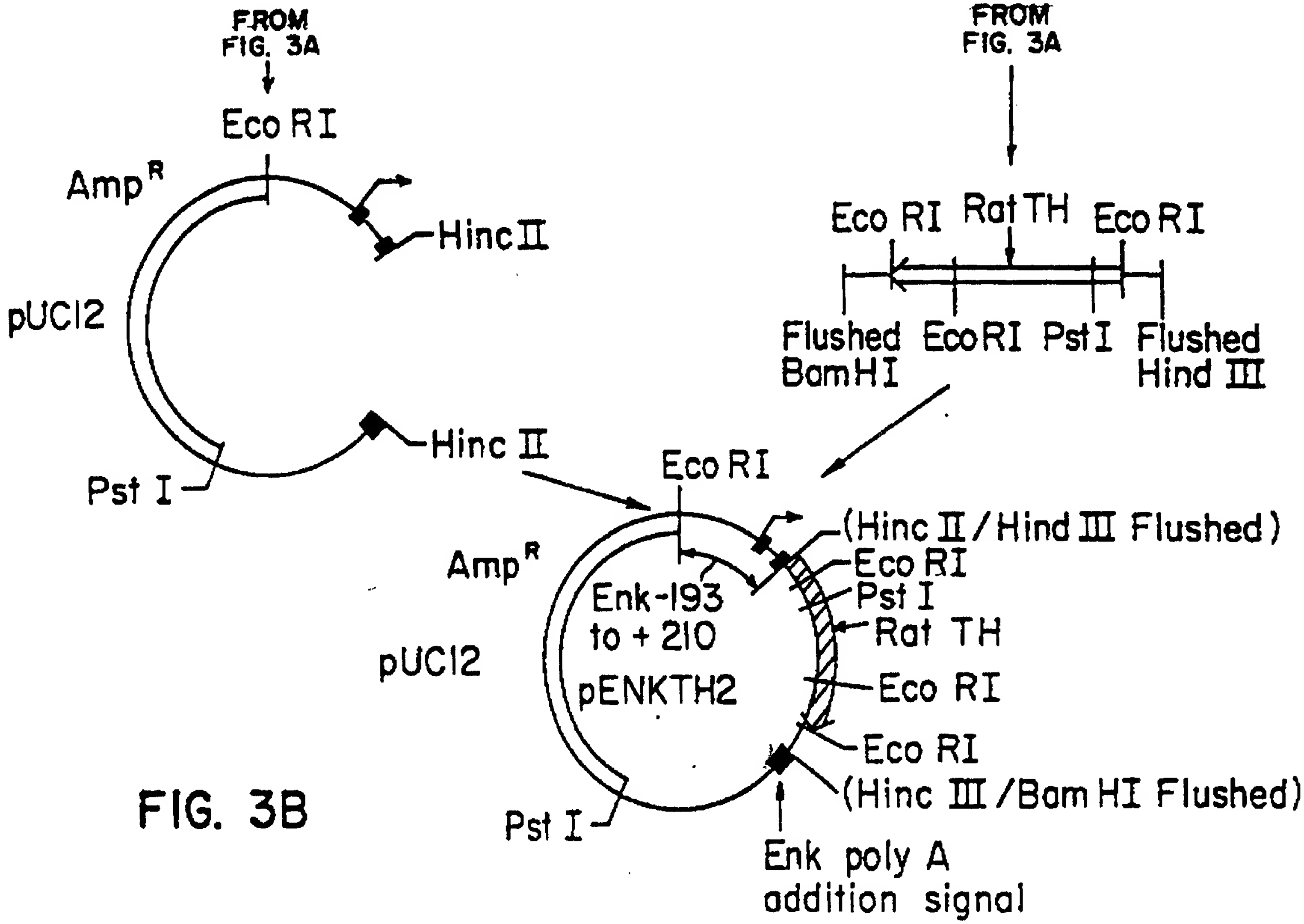

FROM FIG. 3A
Eco RI
Amp^R
HincII
pUC12
Hinc II
Pst I
FROM FIG. 3A
Eco RI
Rat TH
Eco RI
Flushed BamHI
EcoRI
PstI
Flushed Hind III
Eco RI
Amp^R
Enk-193 to + 210
pENKTH2
pUC12
(Hinc II / Hind III Flushed)
Eco RI
Pst I
Rat TH
Eco RI
Eco RI
(Hinc III / Bam HI Flushed)
Pst I
Enk poly A addition signal

FIG. 3B

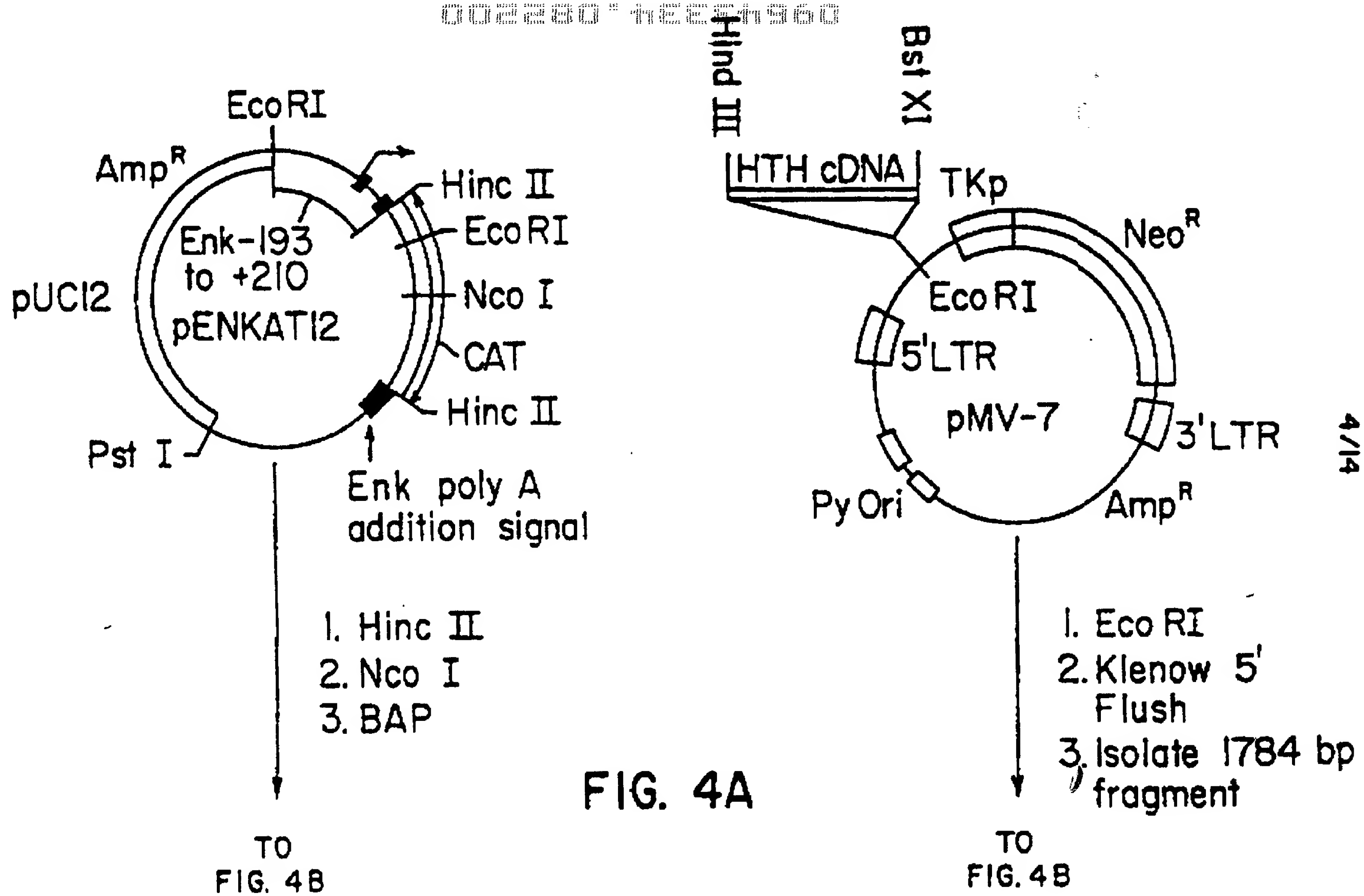


FIG. 4A

SUBSTITUTE SHEET

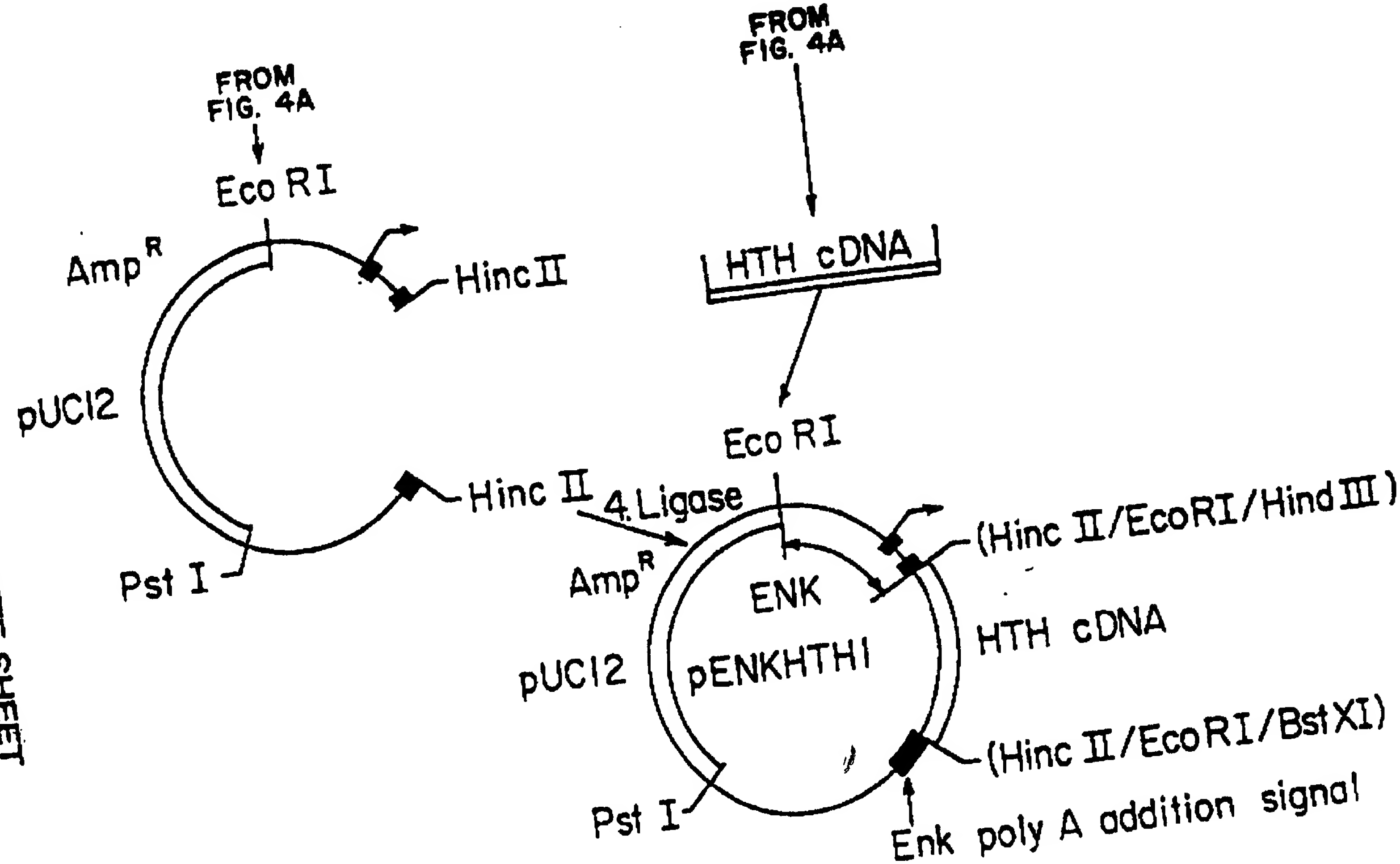
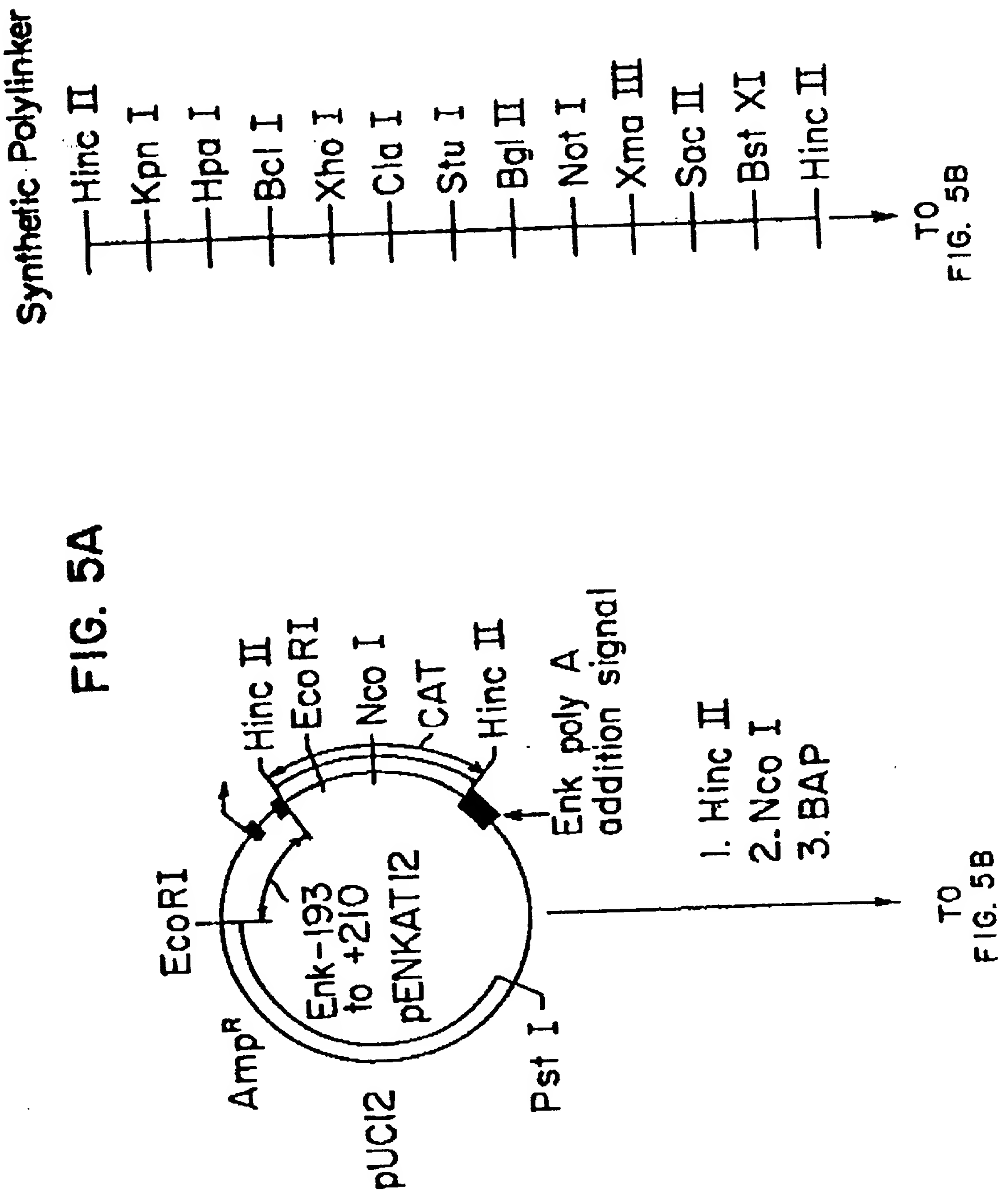

FROM
FIG. 4A
Eco RI
Amp^R
Hinc II
pUC12
Hinc II
Pst I
FROM
FIG. 4A
HTH cDNA
Eco RI
4. Ligase
Amp^R
ENK
(Hinc II/EcoRI/Hind III)
HTH cDNA
pUC12
pENKHTH1
(Hinc II/EcoRI/BstXI)
Pst I
Enk poly A addition signal

FIG. 4B



Synthetic Polylinker
Hinc II
Kpn I
Hpa I
Bcl I
Xho I
Cla I
Stu I
Bgl II
Not I
Xma III
Sac II
Bst XI
Hinc II
TO FIG. 5B
EcoRI
Hinc II
EcoRI
Nco I
CAT
Hinc II
Enk poly A addition signal
Enk-193 to +210
pENKAT12
AmpR
pUC12
Pst I
1. Hinc II
2. Nco I
3. BAP
TO FIG. 5B

FIG. 5A

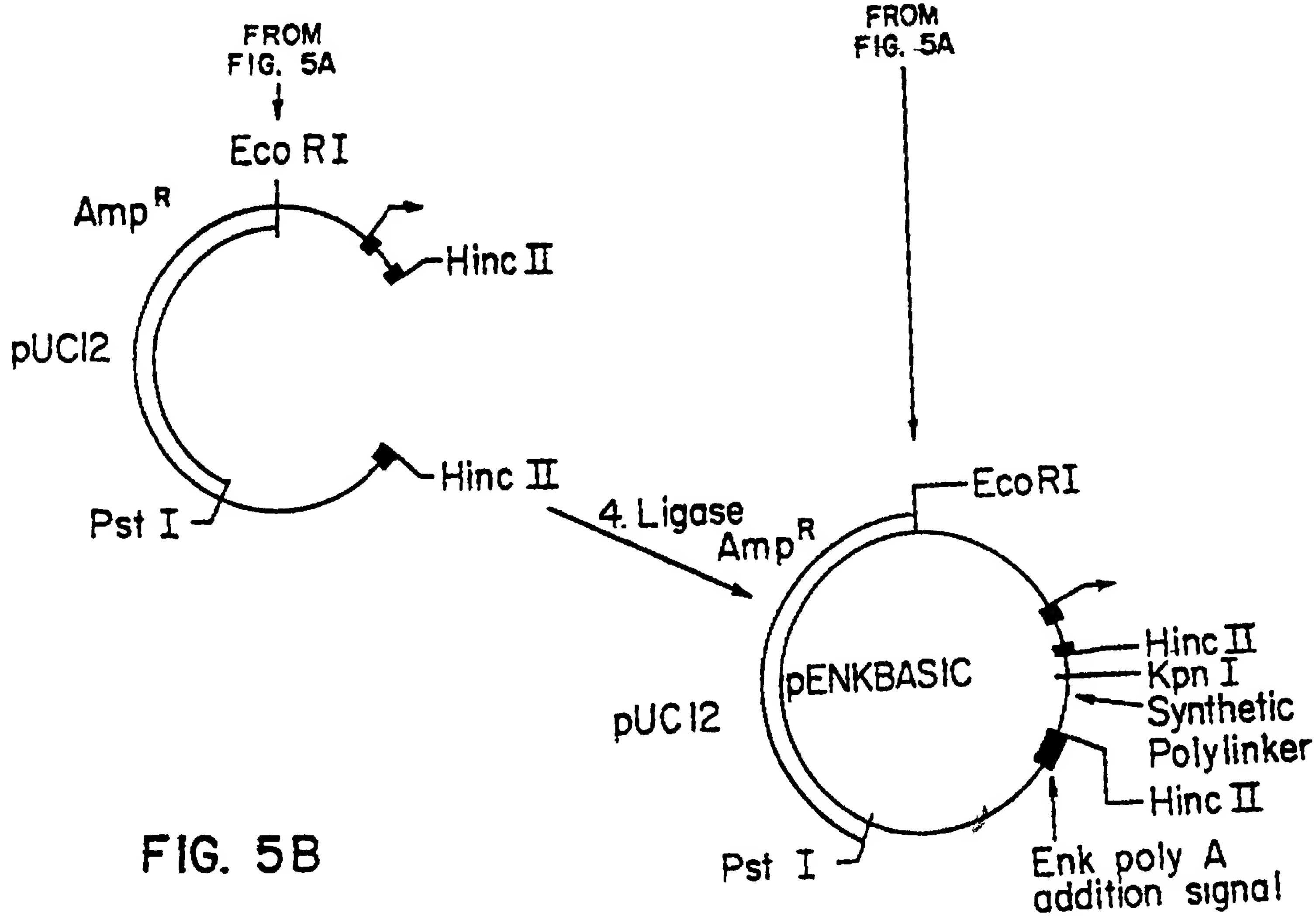
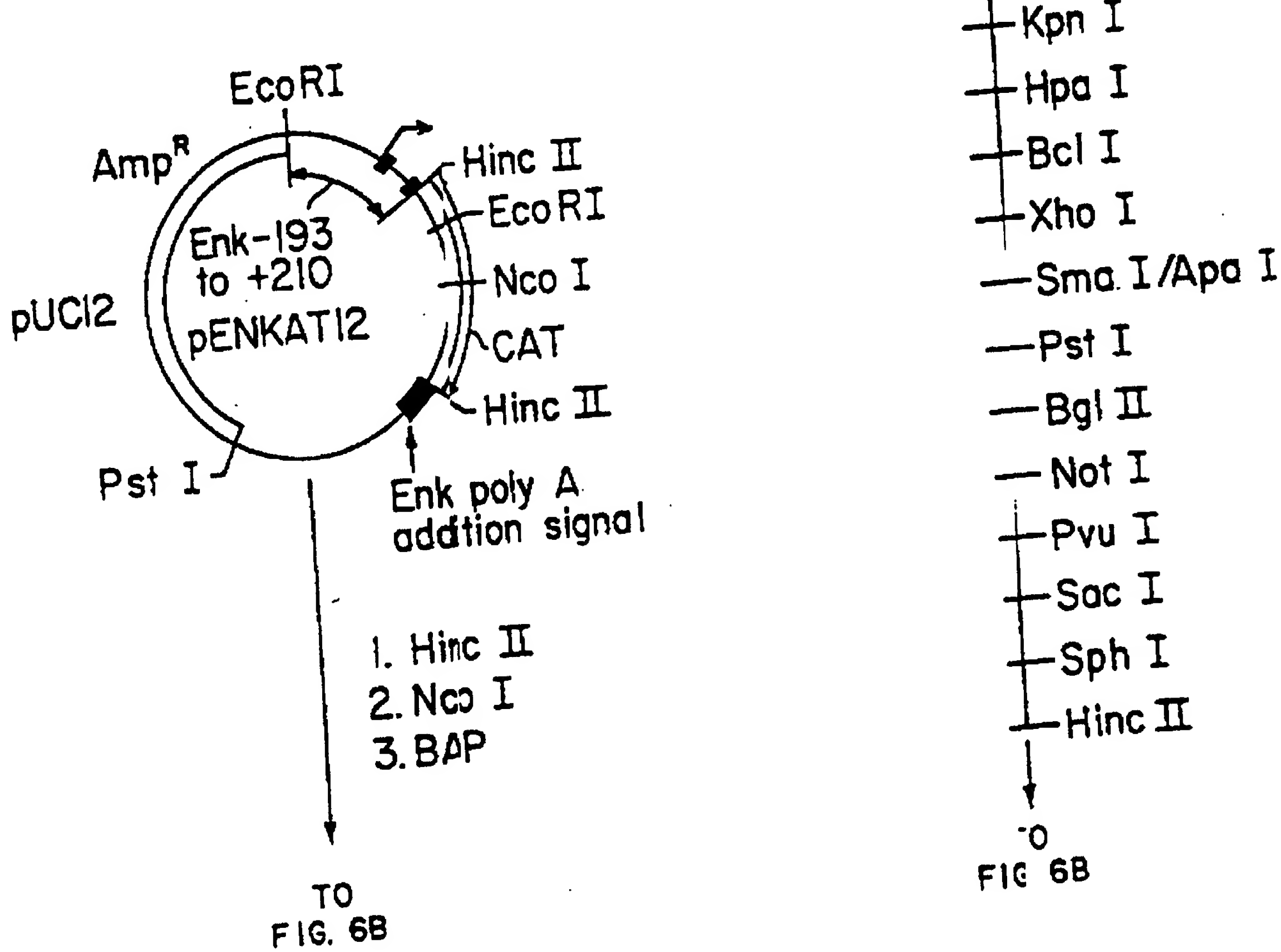

FROM
FIG. 5A
Eco RI
Amp^R
Hinc II
pUC12
Hinc II
Pst I
4. Ligase
FROM
FIG. 5A
EcoRI
Amp^R
pENKBASIC
pUC12
Hinc II
Kpn I
Synthetic
Polylinker
Hinc II
Pst I
Enk poly A
addition signal

FIG. 5B

FIG. 6A


EcoRI
Amp^R
pUC12
Enk-193
to +210
pENKAT12
Pst I
Hinc II
EcoRI
Nco I
CAT
Hinc II
Enk poly A
addition signal
1. Hinc II
2. Nco I
3. BAP
TO
FIG. 6B
Hinc II
Kpn I
Hpa I
Bcl I
Xho I
Sma I/Apa I
Pst I
Bgl II
Not I
Pvu I
Sac I
Sph I
Hinc II
TO
FIG 6B

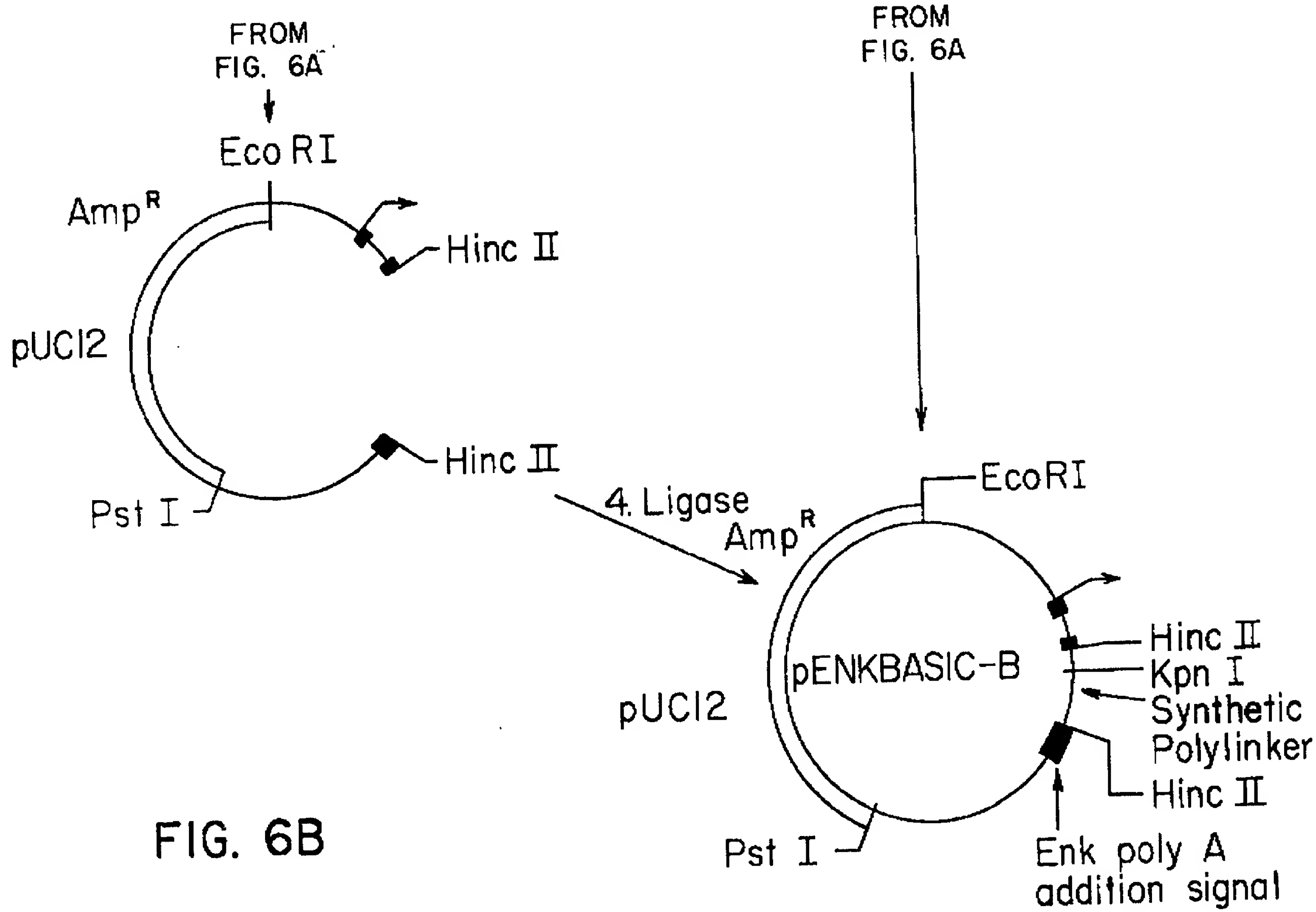
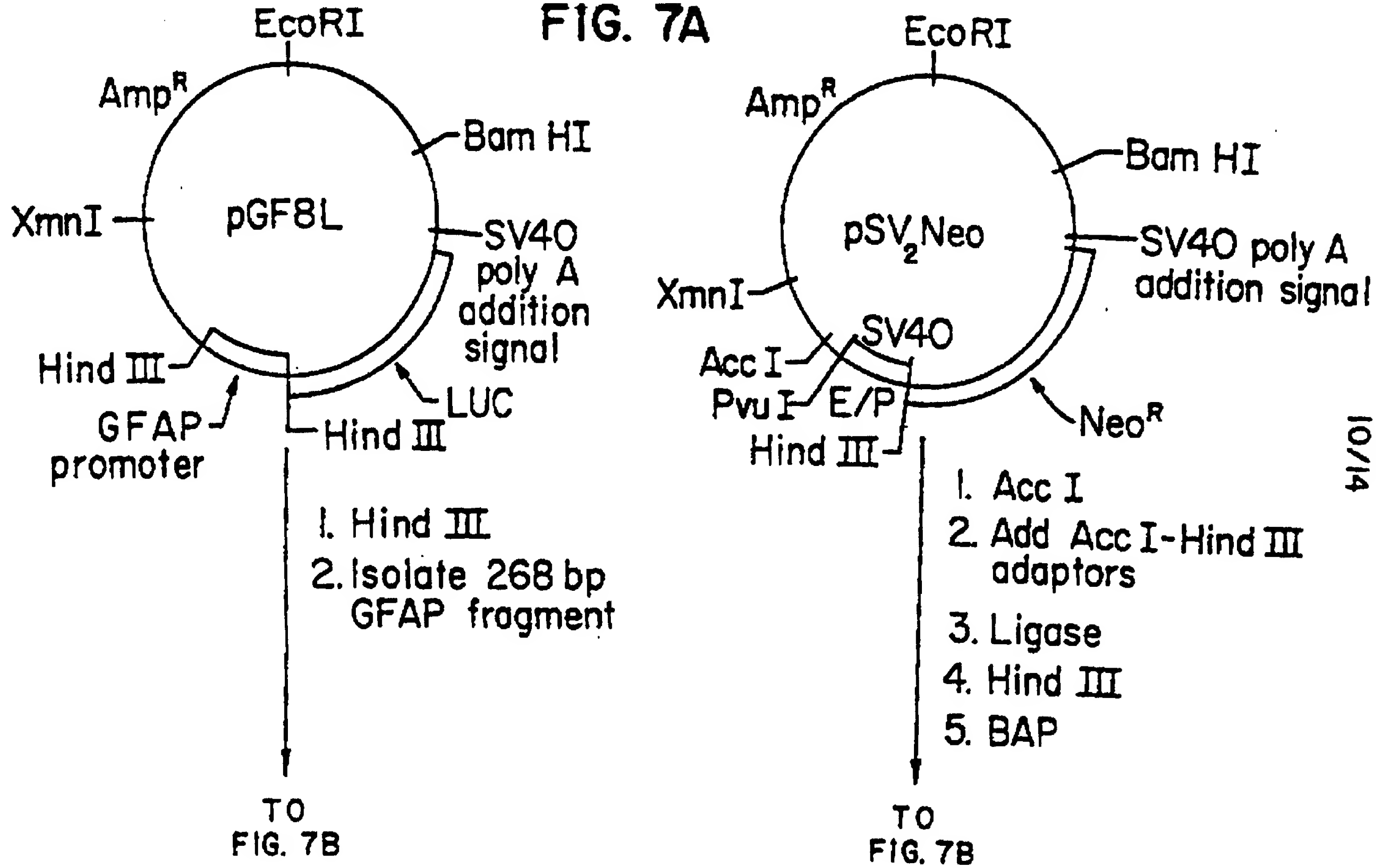

FROM
FIG. 6A
EcoRI
AmpR
Hinc II
pUC12
Hinc II
Pst I
4. Ligase
FROM
FIG. 6A
EcoRI
AmpR
pENKBASIC-B
Hinc II
Kpn I
Synthetic
Polylinker
Hinc II
pUC12
Pst I
Enk poly A
addition signal

FIG. 6B

FIG. 7A


EcoRI
Amp^R
Bam HI
XmnI
pGF8L
SV40 poly A addition signal
Hind III
GFAP promoter
LUC
Hind III
1. Hind III
2. Isolate 268 bp GFAP fragment
TO FIG. 7B
EcoRI
Amp^R
Bam HI
pSV2Neo
XmnI
SV40 poly A addition signal
SV40
Acc I
Pvu I
E/P
Hind III
Neo^R
1. Acc I
2. Add Acc I-Hind III adaptors
3. Ligase
4. Hind III
5. BAP
TO FIG. 7B

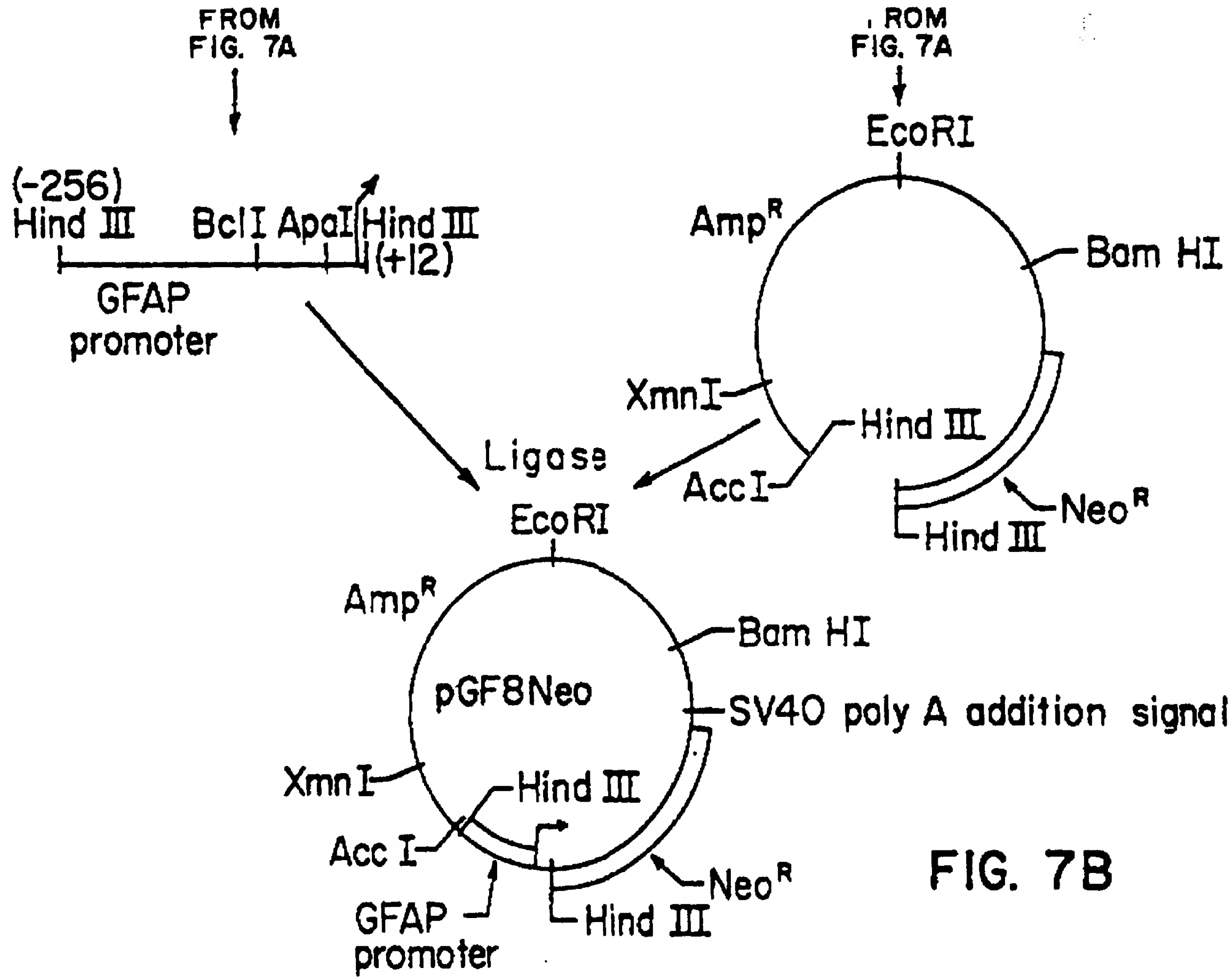

FROM
FIG. 7A
(-256)
Hind III
BclI
ApaI
Hind III
(+12)
GFAP
promoter
ROM
FIG. 7A
EcoRI
AmpR
Bam HI
XmnI
Hind III
AccI
NeoR
Hind III
Ligase
EcoRI
AmpR
Bam HI
pGF8Neo
SV40 poly A addition signal
XmnI
Hind III
Acc I
GFAP
promoter
NeoR
Hind III

FIG. 7B

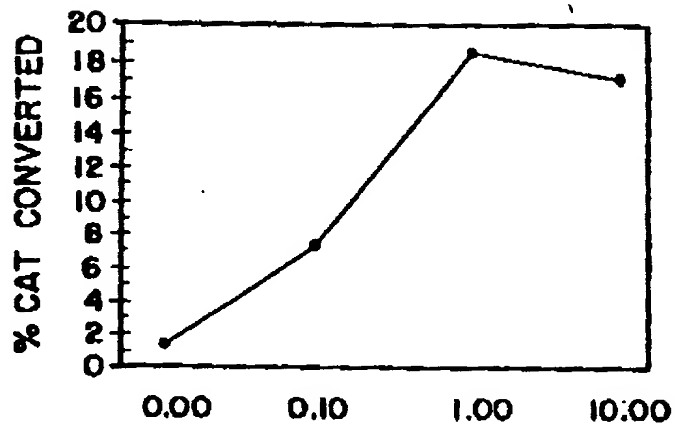

% CAT CONVERTED
20
18
16
14
12
10
8
6
4
2
0
0.00
0.10
1.00
10.00

FIG. 8

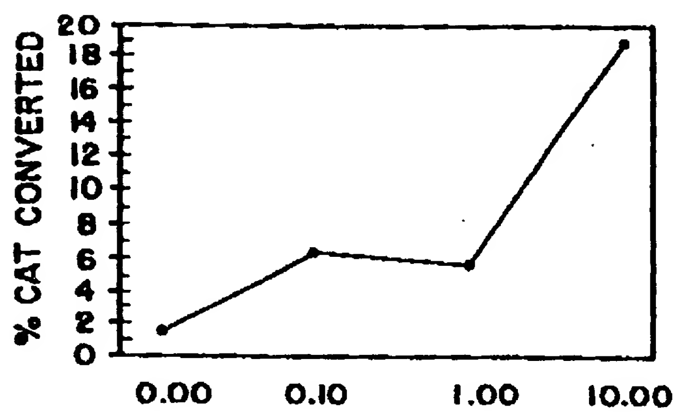

% CAT CONVERTED
20
18
16
14
12
10
8
6
4
2
0
0.00
0.10
1.00
10.00

FIG. 9

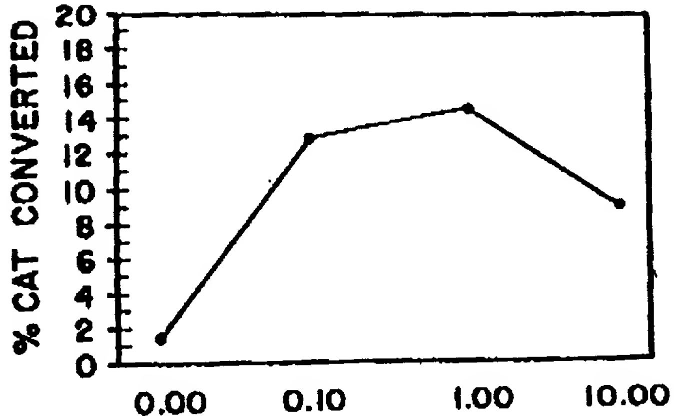

% CAT CONVERTED
20
18
16
14
12
10
8
6
4
2
0
0.00
0.10
1.00
10.00

FIG. 10

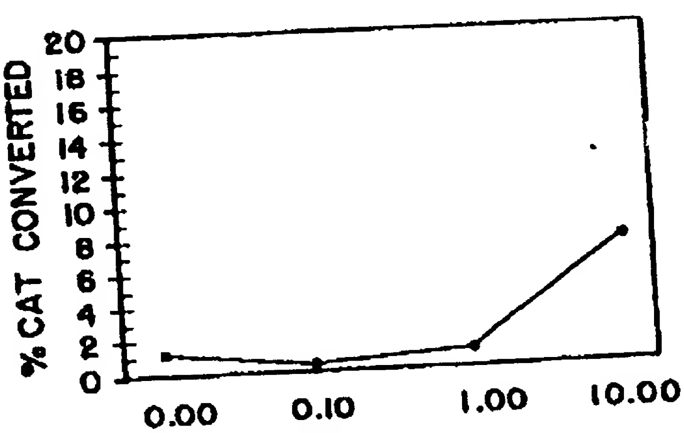
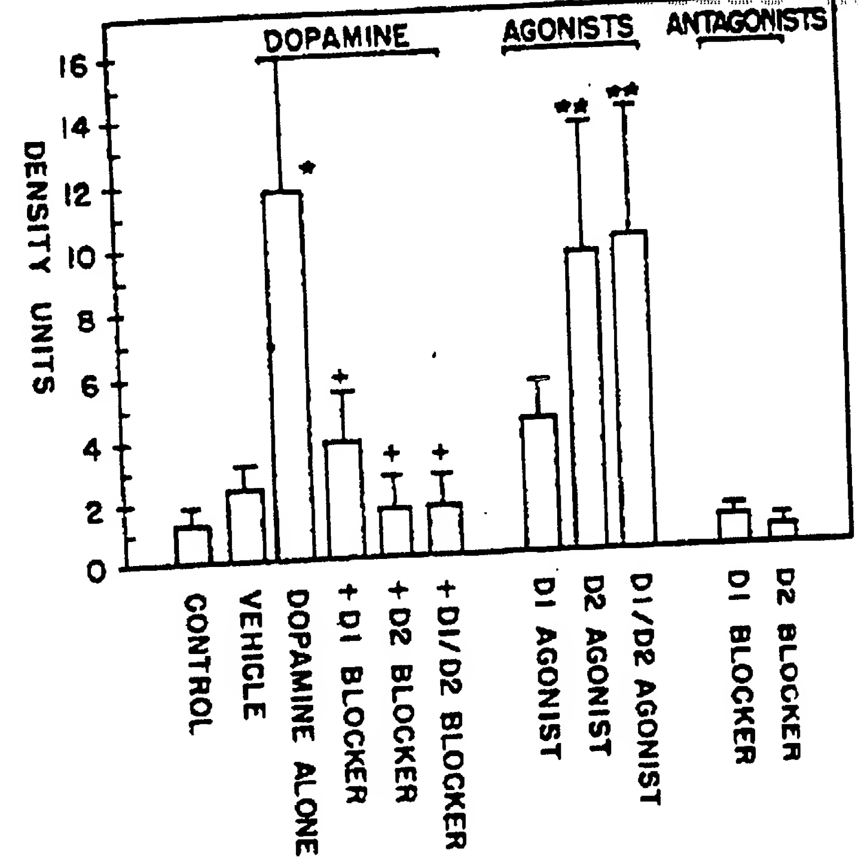

% CAT CONVERTED
20
18
16
14
12
10
8
6
4
2
0
0.00
0.10
1.00
10.00

FIG. 11

FIG. 12



NATIVE RAT PREPROENKEPHALIN mRNA LEVELS
DOPAMINE TREATMENT

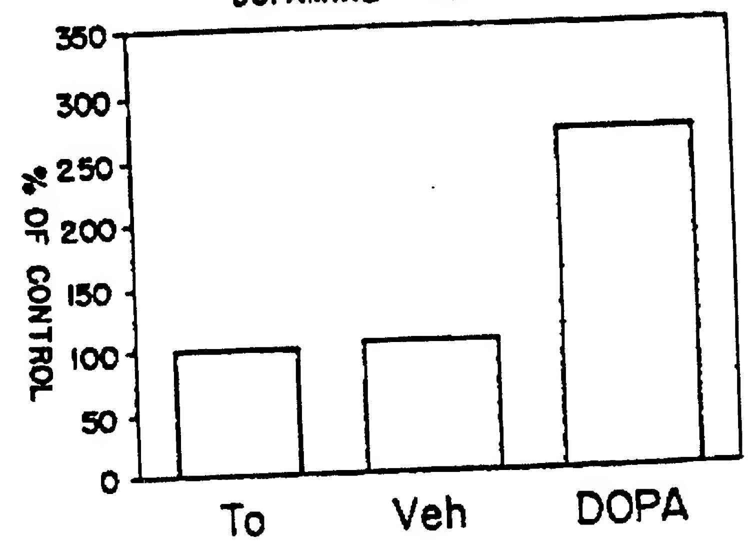


FIG. 13

Docket No. 0097.080

DECLARATION FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled METHOD OF PRODUCING GENETICALLY MODIFIED ASTROCYTES AND USES THEREOF, the specification of which

(check one) is attached hereto.
X was filed on July 6, 1992 as
Application Serial No. ____
and was amended on ____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s) Priority Claimed

(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
none			Yes	No
			Yes	No
			Yes	No

I hereby claim the benefit under Title 35, United States Code, §120 of any United States applications(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)	(Filing Date)	(Status-patented, pending, abandoned)
none		

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Robert E. Heslin, Reg. No. 24,778, Jeff Rothenberg, Reg. No. 26,429, Kevin P. Radigan, Reg. No. 31,789, Susan F. Gullotti, Reg. No. 31,833, Blanche E. Schiller, Reg. No. 35,670, Nicholas Mesiti, Reg. No. 32,782, Susan J. Timian, Reg. No. 34,103, Randall L. Reed, Reg. No. 31,559 and Philip E. Hansen, Reg. No. 32,700

Address all telephone calls to Susan J. Timian, Esq. at telephone no. 518-452-5600
Address all correspondence to Heslin & Rothenberg, P.C.
450 New Karner Road - P.O. Box 12695
Albany, New York 12212-2695

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor Edmund F. La Gamma
Inventor's signature [signature] Date 7/10/92
Residence 2 Ledgewood Circle, Setauket, New York 11733 Citizenship United States
Post Office Address same as above

Full name of second joint inventor, if any Gary Weisinger
Inventor's signature Date
Residence 10 Floyd Lane, Commack, New York 11725 Citizenship Australia
Post Office Address same as above

Full name of third joint inventor, if any Robert E. Strecker
Inventor's signature [signature] Date 7/10/92
Residence 211 Michigan Street, Port Jefferson, New York 11777 Citizenship United States
Post Office Address same as above

Full name of fourth joint inventor, if any Nicholas J. Lenn
Inventor's signature Date
Residence 29 Bayview Avenue, East Setauket, New York 11733 Citizenship United States
Post Office Address same as above

WHEREAS, We, Edmund F. La Gamma, residing at 2 Ledgewood Circle, Setauket, New York 11733, a citizen of the United States of America, Gary Weisinger, residing at 10 Floyd Lane, Commack, New York 11725, a citizen of Australia, Robert E. Strecker, residing at 211 Michigan Street, Port Jefferson, New York 11777, a citizen of the United States of America, and Nicholas J. Lenn, residing at 29 Bayview Avenue, East Setauket, New York 11733, a citizen of the United States of America, have invented certain new and useful improvements in METHOD OF PRODUCING GENETICALLY MODIFIED ASTROCYTES AND USES THEREOF for which we have executed an application for Letters Patent of the United States, U.S. Serial No. 909,281 , filed July 6, 1992, and

WHEREAS, **THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK**, having offices at P.O. Box 9, Albany, New York, 12201-0009, is desirous of obtaining the entire right, title and interest in, to and under the said improvements and the said application;

NOW, THEREFORE, in consideration of the sum of One Dollar ($1.00) to us in hand paid, and other good and valuable consideration, the receipt of which is hereby acknowledged, we the said Edmund F. La Gamma, Gary Weisinger, Robert E. Strecker, and Nicholas J. Lenn have sold, assigned, transferred and set over, and by these presents do hereby sell, assign, transfer and set over, unto said **THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK**, its successors, legal representatives and assigns, the entire right, title and interest in, to and under the said improvements, and the said application and all divisions, renewals and continuations thereof, and all Letters Patent which may be granted thereon and all reissues and extensions thereof, and all applications for Letters Patent which may hereafter be filed for said improvements in any country or countries foreign to the United States, and all Letters Patent which may be granted for said improvements in any country or countries foreign to the United States and all extensions, renewals and reissues thereof; and we hereby authorize and request the Commissioner of Patents of the United States, and any Official of any country or countries foreign to the United States, whose duty it is to issue patents on applications as aforesaid, to issue all Letters Patent for said improvements to the said **THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK**, its successors, legal representatives and assigns, in accordance with the terms of this instrument.

AND WE HEREBY covenant that we have full right to convey the entire interest herein assigned, and that we have not executed, and will not execute, any agreement in conflict herewith.

AND WE HEREBY further covenant and agree that we will communicate to the said **THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK**, its successors, legal representatives and assigns, any facts known to us respecting said improvements, and testify in any legal proceeding, sign all lawful papers, execute all divisional, continuing and reissue applications, make all rightful oaths and generally do everything possible to aid the said **THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK**, its successors, legal representatives and assigns, to obtain and enforce proper patent protection for said improvements in all countries.

IN TESTIMONY WHEREOF, I hereunto set my hand and seal this 10 day of July, 1992.

Edmund F. La Gamma

STATE OF New York)
) ss.:
COUNTY OF Suffolk)

On this 10th day of July, 1992, before me personally came Edmund F. La Gamma, to me known and known to me to be the person of that name, who signed and sealed the foregoing instrument, and he acknowledged the same to be his free act and deed.

Notary Public

JUDITH A. DONOHUE
Notary Public, State of New York
No. 52-4855781, Suffolk County
Commission Expires August 25 1992

IN TESTIMONY WHEREOF, I hereunto set my hand and seal this 17 day of July, 1992.

Gary Weisinger

STATE OF New York)
) ss.:
COUNTY OF Suffolk)

On this 17th day of July, 1992, before me personally came Gary Weisinger, to me known and known to me to be the person of that name, who signed and sealed the foregoing instrument, and he acknowledged the same to be his free act and deed.

Notary Public

JUDITH A. DONOHUE
Notary Public, State of New York
No. 52-4855781, Suffolk County
Commission Expires August 25 1992

REEL 6280 FRAME 514

00230 · 4533 4550

IN TESTIMONY WHEREOF, I hereunto set my hand and seal this 10 day of July, 1992.

Robert E. Strecker

Robert E. Strecker

STATE OF New York)
) ss.:
COUNTY OF Suffolk)

On this 10th day of July, 1992, before me personally came Robert E. Strecker, to me known and known to me to be the person of that name, who signed and sealed the foregoing instrument, and he acknowledged the same to be his free act and deed.

Judith A. Donohue

Notary Public

JUDITH A. DONOHUE
Notary Public, State of New York
No. 52-4865781, Suffolk County
Commission Expires August 28 1992

IN TESTIMONY WHEREOF, I hereunto set my hand and seal this 28 day of August, 1992.

Nicholas J. Lenn

Nicholas J. Lenn

STATE OF)
) ss.:
COUNTY OF)

On this 28 day of August, 1992, before me personally came Nicholas J. Lenn, to me known and known to me to be the person of that name, who signed and sealed the foregoing instrument, and he acknowledged the same to be his free act and deed.

Joan R. Pantorno

Notary Public

JOAN R. PANTORNO
Notary Public, State of New York
No. 4976038
Qualified in Suffolk County
Term Expires 11/24/92